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Link(s) to article on publisher's website:
<http://dx.doi.org/doi:10.21954/ou.ro.000101c0>

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List of Abbreviations

A117V	substitution alanine>valine at codon 117
bp	base pair
BSE	bovine spongiform encephalopathy
°C	Celsius
C	cytosine
CAPS	3-(cyclohesylamino-)1-propanesulfonic acid
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CHO	Chinese hamster ovary
cm	centimetre
CJD	Creutzfeldt-Jakob disease
D177N	substitution aspartate>asparagine at codon 177
dATP	deoxyadenosine-triphosphate
dCTP	deoxycytidine-triphosphate
dGTP	deoxyguanosine-triphosphate
dNTP	deoxyribonucleoside-triphosphate
dTTP	deoxythymidine-triphosphate
dUTP	deoxyuridine-triphosphate
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
Dox	Doxycycline

DTT	dithiothreitol
E200K	substitution glutamic acid>lysine at codon 200
ECL	Enhanced Chemiluminescence
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FBS	foetal bovine serum
FFI	fatal familial insomnia
G	guanine
<i>g</i>	earth's gravitational field
GSS	Gerstmann-Straüssler-Sheinker
h	hour/s
H ₂ O ₂	hydrogen peroxide
hGH	human growth hormone
HRP	horseradish peroxidase
HS	horse serum
IgG	Immunoglobulin G
kDa	kilo daltons
L	liter
M	molar
M128	methionine 128
M145stop	substitution stop codon>methionine 145
M128V	substitution methionine>valine at codon 128
MA	milliAmpere
MBM	meat and bone meal
MEM- α	minimal essential medium- α

Met	methionine
μg	microgram
Min	minutes
ml	milliliter
μl	microliter
mm	millimetre
mM	millimolar
μM	micromolar
moPrP	mouse prion protein
MOPS	3-(N-Morpholino) propanesulfonic acid
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazole-2yl)-2,5-diphenyltetrazolium bromide
NGF	Nerve Growth Factor
ng	nanogram
NFDM	non-fat-dry-milk
nm	nanometers
ORF	open reading frame
P	pellet
P102L	substitution proline>leucine at codon 102
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PNGase F	peptide N-glycosidase F
pg	picogram

PI	propidium iodide
PIPLC	phosphatidylinositol-specific phospholipase C
pmol	picomole
PK	proteinase-K
PMSF	phenylmethylsulphonyl fluoride
rpm	revolution per minute
PFA	paraformaldehyde
PG14	insertion of 9 octarepeat
PrP	prion protein
PrP ^C	cellular prion protein
PrP ^{Sc}	scrapie prion protein
Q217R	substitution glutamine>arginine at codon 217
RNA	ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
s	second
S	supernatant
SEM	standard error of the mean
SDS	sodium dodecylsulphate
SSC	sodium chloride sodium citric acid
TAE	Tris acetic acid + ethylenediaminetetracetic acid
TBS	tris buffer saline
tdT	terminal deoxynucleotidyl transferase
TEMED	N', N, N', N'-tetramethylethylenediamine
Tg	transgenic
T _m	melting temperature
TRE	Tetracycline-Response-element

TSE	Transmissible Spongiform Encephalopathies
TUNEL	tdT-mediated dUTP-X nick end labelling
U	unit
UPR	unfolded protein response
USA	ureidosuccinate
UV	ultra violet
V	Volts
Val	valine
WT	wild-type
w/v	weight/volume
w/w	weight/ weight
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside
Y160	substitution stop codon>tyrosine at codon 160

Acknowledgements

I would like to thank my English supervisor Professor Alun Williams (Department of Pathology and Infectious Diseases, Royal Veterinary College, University of London) for his guidance during my PhD studies. I'm also grateful to Professor Gianluigi Forloni, Head of the Department of Neuroscience who supervised my work.

My special thanks to Dr Roberto Chiesa, Head of the Prion Unit of the Dulbecco Telethon Institute, in the Laboratory of Biology of Neurodegenerative Diseases who guided me in the development of the project and helped me in writing up this thesis. I'm grateful to my colleague Dr Luana Fioriti, who helped me enormously with the preparation of microglia cultures and with the toxicity experiments.

A special thanks to Felice e Vincenzo De Ceglie for digital scanning films and pictures.

I'm grateful to Professor Roberto Sitia and Dr Luigina Tagliavacca, who work at the San Raffaele Scientific Institute, DIBIT, Milan, for advise with ER stress analysis and for providing pTZ/moCHOP, BiP and GADPH cDNAs, anti-giantin and anti-calnexin antibodies.

A special thanks to Dr Hans Jornvall from Karolinska Institute, Stockholm, Sweden, for providing PC12 Tet-on and CHO-AA8-Luc cells. I thank Dr David Harris, from Washington University, St. Louis, Missouri for providing mouse PrP constructs 3F4 tagged and P45-66 antibody. I would also like to

thank Dr Richard Kascsak, from NYS, New York, for providing 3F4 antibody and Dr. Jan P.M. Langeveld, from CIDC-Lelystad, for providing 98A3 antibody.

Finally I would like to thank the Fondazione Monzino and Carrefour Foundation, the European Commission and Telethon for funding my research.

Summary

Inherited prion diseases are linked to mutations in the prion protein (PrP) gene that are presumed to favor conversion of PrP into a neurotoxic isoform (PrP^{Sc}). Several cellular models of inherited prion diseases have been developed in which mutant PrP constitutively expressed acquires PrP^{Sc}-like properties but is not cytotoxic. However, the use of constitutive models does not exclude the potential toxicity of mutant PrP, as it can be speculated that only clones resistant to PrP toxicity are selected after clonal selection. To rule out this possibility a tetracycline-inducible (Tet-on) model was developed in this thesis, in which PrP expression is switched on after clonal selection.

cDNAs encoding mouse wild-type PrP, as well as mouse PrP homologues of the human D178N (D177N/M128 and D177N/V128) and nine-octapeptide (PG14) mutations under the control of the tetracycline-responsive element, were transfected in PC12 Tet-on cells. The Tet-on system was chosen based on the lack of pleiotropic or toxic effects and because it allows a tightly regulated expression of the protein of interest. To ensure unwanted background expression of PrP in the absence of inducer, cells were co-transfected with pTet-tTS, encoding the tet-controlled transcriptional silencer.

The D177N and PG14 mutants expressed in this system displayed biochemical properties reminiscent of PrP^{Sc}, including detergent insolubility and low protease resistance. Low levels of mutant PrPs were detected on the cell surface by confocal immunofluorescence analysis compared to wild-type PrP.

These mutant displayed reticular intracellular distribution, suggesting impaired delivery to the cell surface and retention in the endoplasmic reticulum (ER).

Evaluation of cellular viability revealed a decrement in cell survival after 96 h of mutant PrP expression, associated with an increased number of apoptotic cells. This effect was not fully consistent and was not exacerbated by neuronal differentiation with NGF or by treating cells with H₂O₂, tunicamycin, or by removing serum. The results presented in this thesis also demonstrate that expression of PrP does not protect PC12 Tet-on cells from such stresses.

To explore the possibility that expression of mutant PrPs triggered toxic response pathways in the ER, the mRNA level of ER stress markers was measured. The results shown in this thesis indicate that, although the altered intracellular distribution of mutant PrPs suggested retention of the protein in the endoplasmic reticulum, ER stress responses were not detected.

Chapter 1: Introduction

1.1 General introduction

1.1.1 Prion diseases

Prion diseases, also called Transmissible Spongiform Encephalopathies (TSEs), are fatal neurodegenerative disorders that have attracted enormous attention because they have been proposed to exemplify a novel mechanism of biological information transfer based on the transmission on protein conformation, rather than on a nucleic acid sequence (Prusiner, 1982). This group of diseases includes Kuru, Creutzfeldt-Jakob Disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI) in humans (Table I.I), as well as scrapie in sheep and goats, Bovine Spongiform Encephalopathy (BSE) in cattle, and encephalopathies in mink (Marsh and Hadlow, 1992), cats (Leggett et al., 1990), mule deer, Rocky Mountain elk (Williams and Young, 1980; Williams and Young, 1982), and several exotic ungulates (Kirkwood and Cunningham, 1994; Taylor and Woodgate, 1997) (Table I.II).

Table I.I: Human prion diseases

Disease	Mechanism of pathogenesis
Kuru (Fore people)	Infection through ritualistic cannibalism
Iatrogenic Creutzfeldt-Jakob	Infection: <ul style="list-style-type: none"> A) <u>Peripheral transmission</u>: <ul style="list-style-type: none">Prion-contaminated human growth hormone (hGH)Prion-contaminated gonadotrophin B) <u>Central transmission</u>: <ul style="list-style-type: none"> Prion-contaminated Dura mater Prion-contaminated Cornea Prion-contaminated Surgical instruments Prion-contaminated Cortical EEG electrodes
Variant Creutzfeldt-Jakob	Infection from BSE – contaminated food
Sporadic Creutzfeldt-Jakob	Somatic mutation in the PrP gene? Spontaneous conversion of PrP ^C into PrP ^{Sc} ?
Familial Creutzfeldt-Jakob	Germline mutations in the PrP gene
Gerstmann-Sträussler-Scheinker Syndrome	Germline mutations in the PrP gene
Fatal Familial Insomnia	Germline mutations in the PrP gene
Sporadic Fatal Insomnia?	Somatic mutation in the PrP gene? Spontaneous conversion of PrP ^C into PrP ^{Sc} ?

Table I.II: Animal prion diseases

Disease	Animal affected	Mechanism of pathogenesis
Scrapie	Sheep, Goats	Infection in susceptible sheep
Bovine spongiform encephalopathy	Cattle	Infection with prion contaminated meat-bone meal
Trasmissible mink encephalopathy (Marsh and Hadlow, 1992)	Mink	Infection with prions from sheeps or cattle
Chronic wasting disease (Williams and Young, 1980; Williams and Young, 1982)	Mule deer, white tailed dear Rocky Mountain elk	As sheep
Feline spongiform encephalopathy (Kirkwood and Cunningham, 1994)	Cats, pumas, tigers, lions, cheetahs, ocelots	Infection with prion contaminated meat-bone meal
Exotic ungulate encephalopathy (Kirkwood and Cunningham, 1994; Taylor and Woodgate, 1997)	Nyala, oryx, greater kudu, eland, gemsbok, American bison, Ankole cattle	Infection with prion contaminated meat-bone meal

The chemical nature of the infectious agent in TSEs has been the subject of intense scrutiny for nearly 40 years. Many lines of evidence indicate that an altered form of a cellular protein (named prion protein, PrP) plays a key role in the origin of these diseases. It accumulates in the brain upon conformational change, without modifications of the primary structure. According to the prion hypothesis (see section 1.2), this altered form (called PrP^{Sc}) would be able to self-propagate by impressing its abnormal conformation on normal cellular PrP (PrP^C) molecules (Cohen et al., 1994). An alternative hypothesis states that there may be an undiscovered viral-like agent containing a nucleic acid genome that is responsible for transmission (Chesebro, 1997).

Three different manifestations of prion diseases are known: infectious, familial and sporadic. The infectious origin is dramatically illustrated by Kuru, a neurodegenerative illness of the Fore and related tribes of New Guinea that was found to be transmitted by ritual cannibalism (Gajdusek and Zigas, 1957). Other examples include iatrogenic cases of CJD due to dura mater grafts and therapeutic administration of cadaver-derived hormone (Brown et al., 1992) (for the description of human prion diseases see section 1.4), as well as BSE and its human counterpart "variant" CJD .

Bovine spongiform encephalopathy was first described in the United Kingdom (UK) (Jeffrey et al., 2001; Jeffrey and Wells, 1988; Wells et al., 1987) in 1986. In June a case of spongiform encephalopathy was discovered in a Nyala (a South African antelope which belongs to the family of Bovidae, subfamily of Bovinae) which had been kept at Marwell Zoo (Jeffrey et al., 2001; Jeffrey and Wells, 1988) and in December Dr Gerald Wells reported the first case of spongiform encephalopathy in a cow (Wells et al., 1987). In the following years increasing numbers of cases were diagnosed in cows, reaching

epidemic proportions and causing the death of now almost 180,000 animals. Other cases of bovine spongiform encephalopathy were also diagnosed in the UK in other captive Bovidae through 1986 and 1992 (Kirkwood et al., 1990). Epidemiological studies revealed that the use of meat and bone meal (MBM) as a high protein supplement feed might be the vehicle of infection (Wilesmith et al., 1988). MBM was produced from rendering the waste parts of mixed species including sheep and cattle that were not used for human consumption. It was hypothesised that once the causative agent of BSE had been introduced into the rendering process it would have been recycled to infect other cattle through MBM. It was further postulated that BSE occurred in the UK first, rather than in any other country, because of the ratio of sheep to cattle tissue going into rendering in the UK. To eradicate the disease, in 1988 the government introduced the "Feed Ban", which banned the feeding of ruminant proteins to ruminants. The effect was not immediately apparent because of long incubation period; however by 1992 the number of cases fell by about 40% a year on average (Smith and Bradley, 2003). However, it turned out that this ban was insufficient in totally preventing further cases of BSE and a stricter feed ban was introduced in August 1996. This new ban entirely prohibited cattle parts being rendered into feed, including feed for chicken and pigs. Factors underlying this 1996 ban were that there were cross-contaminations in feed-factories that produced both cattle and pig/chicken feed and farmers frequently did not differentiate between cattle, pig and chicken feed. This such a measure was necessary because cattle protein, even if used legally in chicken and pig feed, inevitably ended up being fed to cattle as well. The few cases of BSE reported in animals born after 1 August 1996 are known as "BARB" (Born After the Reinforced feed Ban). To explain the persistency of the disease different

hypothesis were made: 1) some animals might have been exposed to BSE through maternal transmission; 2) some animals might have been exposed to BSE through feed carried over before 1 August 1996; 3) there might be routes of transmission which have not yet been identified; 4) the disease may occur spontaneously.

Initially BSE was regarded as a problem confined to UK, but there were soon indications that other countries to which cattle, cattle products and MBM had been exported were also involved. The first BSE cases outside the UK were diagnosed in the Falkland Islands and Oman in 1989, in animals imported from the UK. The first cases in cattle that were born and raised outside the UK were reported in the Republic of Ireland in 1989, in Switzerland in 1990 and in France in 1991. In 1997, the first cases were diagnosed in Belgium, the Netherlands and Luxemburg (Heim and Wilesmith, 2000). Other countries with native BSE cases are now Portugal, Spain, Liechtenstein and Japan. Imported and native cases have been diagnosed in Canada, Denmark, Germany and Italy (Table I.III) (Smith and Bradley, 2003) (data from Office Internationale des Epizooties, OIE).

In 1996 the occurrence of a variant form of CJD (vCJD) was reported in UK, affecting young people and having consistent and unique clinicopathological pattern, including the presence of florid plaques extensively distributed in the cerebrum and cerebellum (Will et al., 1996). As of April 5, 2004, a total of 156 (definite or probable) cases of vCJD had been reported worldwide: 146 in the UK, 6 in France, one each from Canada, Ireland, Italy and United States. The observation that the majority of the patients (excluding the French and the Italian cases) had multiple-year exposures in the UK between 1986 and 1996 raised concerns that BSE could have spread to humans, by dietary exposure. The infection of French and Italian patients must have come either from beef imported from the UK or from French BSE affected cattle.

Experimental evidence has provided compelling evidence of a link between BSE and vCJD. Brain material from bull BSE (cattle) and vCJD (human patients) produce the same profile of disease when inoculated in mice, based on the incubation time, the clinical symptoms, the type of lesions (Bruce et al., 1997; Hill et al., 1997a) and the glycoform pattern of protease-resistant PrP (Collinge et al., 1996); 2) neuropathologically, vCJD lesions are similar to lesions found in macaques inoculated with BSE (Lasmezas et al., 1996b).

Familial prion diseases, which include about 15% of the cases of CJD, all cases of GSS and almost all cases of FFI are inherited in an autosomal fashion and are linked to germline mutations in the PrP gene (Young et al., 1999). According to the prion hypothesis (see section 1.2) mutations destabilise the conformation of the protein and favour spontaneous conversion to PrP^{Sc} (Cohen et al., 1994). Sporadic prion diseases, which include most cases of CJD, have no obvious infectious or genetic aetiology and has a near uniform prevalence worldwide. The origin of the disease can be explained by spontaneous

conversion of wild-type PrP to the PrP^{Sc} state without necessity for contact with exogenous prions or alternatively by rare somatic mutations in the PrP gene. In the viral theory, sporadic forms could be due to an ubiquitous infection and PrP mutations might increase susceptibility to disease (Chesebro, 1997).

Familial and sporadic prion diseases, as well as infectious forms can be experimentally transmitted to laboratory animals, suggesting the ability of PrP^{Sc} to propagate infection (Gajdusek et al., 1966; Gajdusek and Gibbs, 1971; Gajdusek and Gibbs, 1972; Gajdusek et al., 1967; Gibbs et al., 1968).

1.1.2 The cellular prion protein

The cellular prion protein (PrP^C) is a 33-35 kDa glycoprotein, linked to the outer leaflet of the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor (Diener, 1987; Kretzschmar et al., 1986b; Stahl et al., 1987). PrP^C is encoded by a small single-copy housekeeping gene that in humans is located on chromosome 20 (Kretzschmar et al., 1986b). In man, the gene has only three exons and the entire open reading frame (ORF) is in one exon. The 2.5 kb transcript is expressed at high levels in the brain, spinal cord and at lower levels in other peripheral tissues, including lung, skeletal muscle, heart and lymphoid tissues (Bendheim et al., 1992; Kretzschmar et al., 1986a). In the central nervous system (CNS), PrP is expressed in neurons, ependymal cells, choroid plexus epithelium, endothelial cell, meninges and astrocytes (Brown et al., 1990). PrP mRNA is first detectable in the brains of mice beginning in early embryogenesis, and its level increases as development proceeds (Manson et al., 1992).

The primary structure of PrP^C is highly conserved across mammalian species and contains several distinct domains, including an N-terminal signal

peptide, a series of five proline- and glycine-rich octapeptide repeats, a central hydrophobic segment and a C-terminal hydrophobic region (Figure 1.1). Nuclear magnetic resonance analysis (NMR) of recombinant PrP^C from various species has revealed a C-terminal globular structure and an N-terminal flexible, unstructured region (Figure 1.2). The globular structure is composed of three α -helices (two of which are linked by a disulphide bridge) and two short anti-parallel β -pleated segments (Riek et al., 1997).

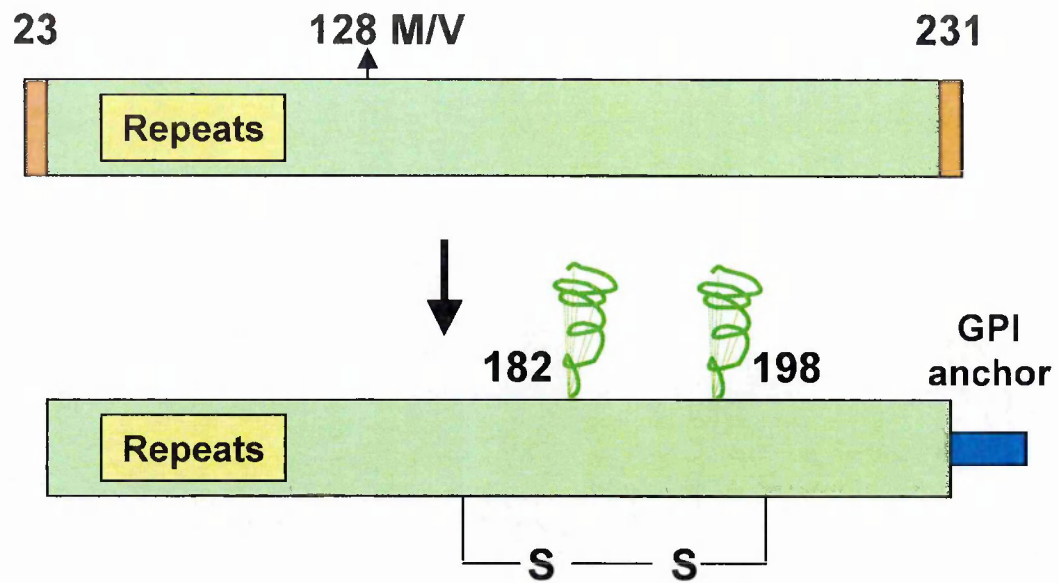


Figure 1.1: Post translational modifications of mouse PrP.

The translational product of 254 aminoacids is cleaved at the N-terminus and at the C-terminus. A GPI anchor is added to the serine 231 and N-glycosilation occurs at residues 182 and 198. A disulphide bridge is formed between cysteine residues 180 and 215. Repeats region, spanning residues 51-91, and Met/Val 128, a common polymorphism on Caucasian population, are represented.

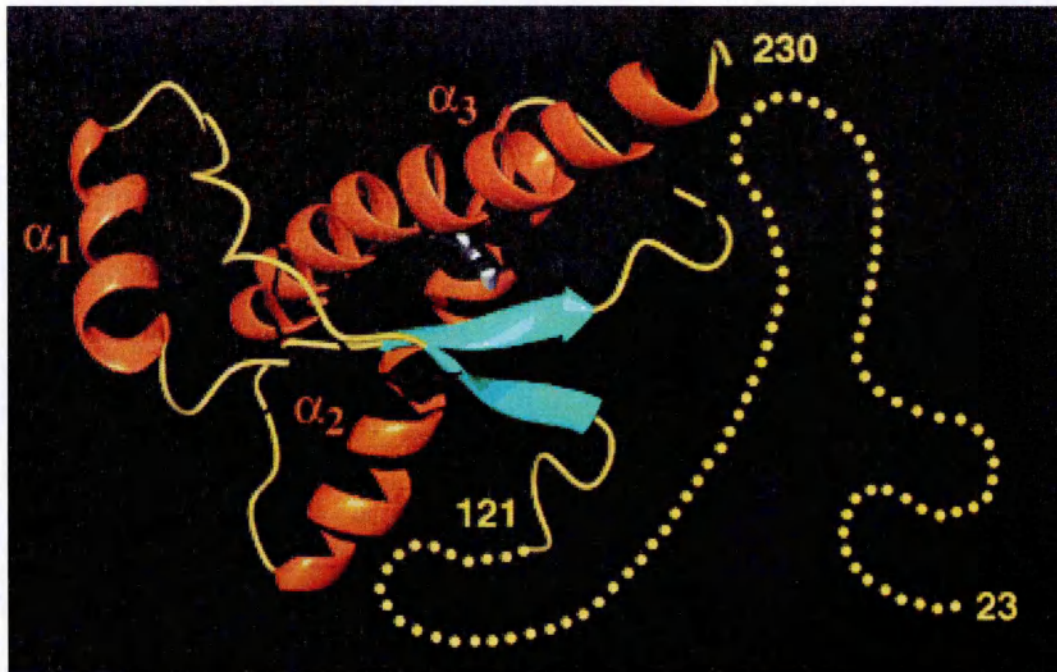


Figure 1.2: Three-dimensional structure of moPrP (23-231).

NMR analysis of recombinant moPrP reveals an N-terminal random coil polypeptide (23-123) and a C-terminal globular structure. The globular structure is composed of three α -helices (residues 144-154, 175-193, 177-219), a short segment of helix-like structure residues (222-226) and two stranded antiparallel β -sheet (residues 128-131, 161-164).

Like other membrane proteins, PrP is synthesized in the endoplasmic reticulum (ER) and transits the Golgi on its way to the cell surface. The murine PrP^C protein is synthesised as a 254 amino acids polypeptide chain and is subject to several posttranslational modifications: the signal peptide of 22 aminoacids is cleaved at the N-terminus, and the hydrophobic signal of 23 amino acid is removed from the C-terminus on addition of the GPI anchor when in transit through the ER (Stahl et al., 1987; Turk et al., 1988). Asparagine-linked glycosylation (N-glycosylation) occurs at residues 182 and 198 in a loop formed by the disulfide bond between cysteine residues at positions 180 and 215 (Figure 1.1) (Caughey et al., 1989). The N-linked oligosaccharides added initially in the ER are of the high mannose type and are sensitive to digestion by endoglycosidase H; these are subsequently modified in the Golgi to yield complex type chains that contain sialic acid and are resistant to endoglycosidase H digestion (Safar et al., 1990; Stahl et al., 1992). At steady state, different forms of PrP can be detected by Western Blot, corresponding to the fully glycosylated, mono-glycosylated and unglycosylated forms. In addition, PrP contains two independent functional nuclear localisation signals (NLS) in the N-terminal domain (Gu et al., 2003a). Nuclear forms have been described for PrP carrying the stop-mutations at codon 145 and 160 (Lorenz et al., 2002; Zanusso et al., 1999).

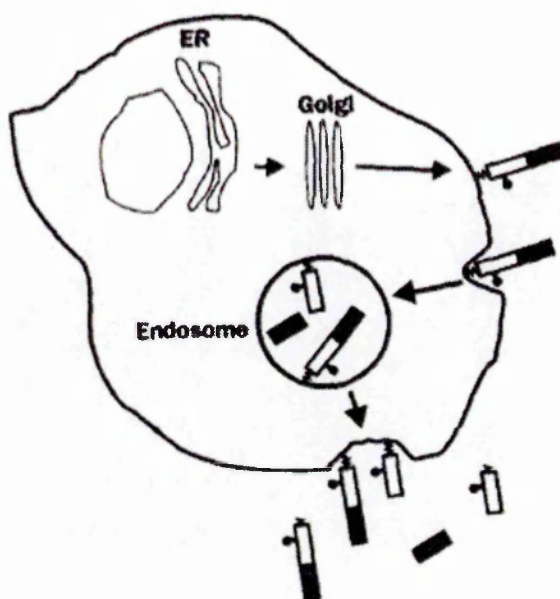
Also in common with other GPI-anchor proteins PrP^C is transported to the cell surface where it is predominantly located in detergent resistant microdomains, rich in sphingolipid and cholesterol, known as rafts or caveolae (Gorodinsky and Harris, 1995; Peters et al., 2003; Vey et al., 1996). Saturated acyl chains covalently linked to the protein are sufficient for the raft association of GPI-anchor proteins, where the acyl chains of the anchor interact with lipids

of the rafts (Simons and Toomre, 2000). However, in the case of PrP, the N-terminus of PrP and not the GPI anchor, acts as a cellular raft targeting determinant, since raft association was abolished by the deletion of the N-terminal region (residues 23-90) (Baron and Caughey, 2003; Walmsley et al., 2003). After its delivery to the cell surface, the protein constitutively cycles between the plasma membrane and an endocytic compartment, with a transit time of ~60 min (Shyng et al., 1993) (Figure 1.3A).

PrP^C undergoes different post-translational cleavages as part of its normal metabolism (Figure 1.3B). Both cleavages occur relatively slowly in comparison to the half life of the protein, so that at steady state several different cleavage products in addition to the intact protein can be detected. One cleavage occurs within the GPI-anchor (Borchelt et al., 1993; Harris et al., 1993; Parkin et al., 2004) and releases the polypeptide chain into the extracellular milieu. The cellular localization in which this cleavage occurs is unknown, although for other GPI-anchored proteins which undergo a similar cleavage it has been speculated that a cell surface phospholipase is responsible (Low, 1989). The fact that the formation of this form of PrP is stimulated by the raft-disrupting agent filipin confirms the hypothesis that the cleavage occurs on the cell membrane (Parkin et al., 2004). A second cleavage involves the action of a metalloprotease that acts very near to the GPI anchor and sheds PrP from the cell surface (Borchelt et al., 1993; Parkin et al., 2004). When PrP is internalised in the endocytic compartment some of the molecules are cleaved at position 111-112 (α -site) by metalloenzymes producing a C-terminal fragment named C1 carrying the sugar chains and a complementary N-terminal fragment (N1) (Chen et al., 1995; Shyng et al., 1993; Vincent et al., 2000; Vincent et al., 2001). Full length PrP and the C1 fragment are re-exposed on cell membrane, while

the N1 fragment is secreted into the extracellular milieu (Shyng et al., 1993; Vincent et al., 2000). Finally, in prion infected cells and brains, a longer C-terminal fragment starting from around residue 90 (β -site), named C2 accumulates (Caughey et al., 1989; Chen et al., 1995; Yadavalli et al., 2004). The accumulation of this form in scrapie infected cells is inhibited by calpain inhibitors, suggesting an active role of these proteases in the formation of C2. This fragment is also generated in small amounts in normal brain and in cell lines expressing endogenous PrP, from which it is also possible to recover the complementary N-terminal fragment, called N2 (Jimenez-Huete et al., 1998; Mange et al., 2004). Interestingly, the cleavage of PrP in the octapeptide region was observed after exposure to reactive oxygen species in the presence of divalent copper ions (Mange et al., 2004; McMahon et al., 2001). On the basis of these data it has been suggested that modulation of oxidative stress can be linked to the N-terminal cleavage of PrP (Mange et al., 2004). In this hypothesis, the C2 terminal fragment may be important for the function of PrP (through, for example, signal transduction pathways). Then the second cleavage, in the α -site, would inactivate the biological active form (Mange et al., 2004).

A



B

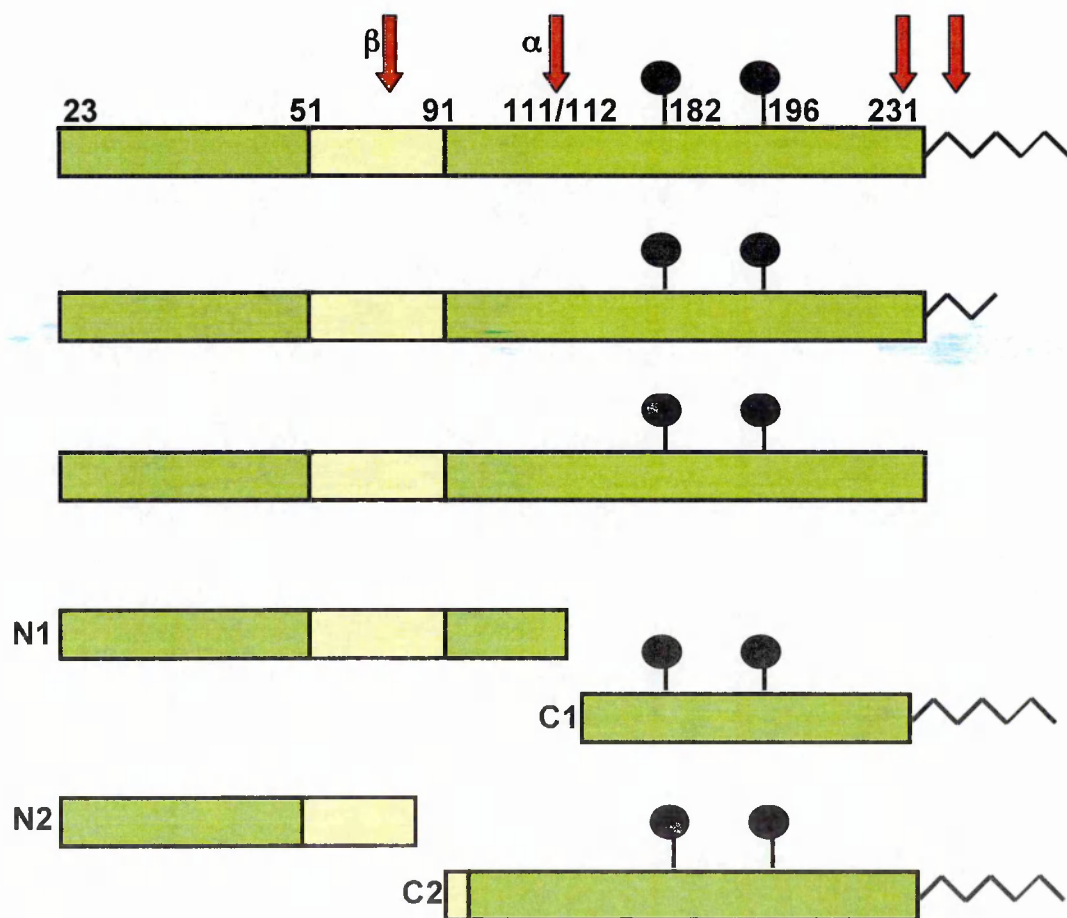


Figure 1.3: Processing of PrP^C.

(A) Model for the endocytic targeting of PrP. After reaching the cell surface, PrP is internalized into an endocytic compartment from which most of the molecules are recycled intact to the cell surface. A small percentage of the endocytosed molecules are proteolytically cleaved, producing N-terminal and C-terminal fragments which are subsequently externalised. Some of the membrane-anchored PrP molecules are released into the extracellular medium by cleavage within or near the GPI anchor.

(B) Representation of moPrP and its fragments. N-glycosylation at residues 182 and 196 and the GPI anchor, attached to the serine 231 are represented. Octapeptide region (residues 51-91) is shown in yellow. PrP^C undergoes different post-translational cleavages. Two different cleavages can occur within or very near the GPI anchor after PrP has reached the cell surface, so that the polypeptide chain is released in the extracellular milieu. The cleavage at the position 111-112 (α -site) occurs in an endocytic compartment and determines the formation of two fragments: a N-terminal (N1) and a C-terminal (C1) which is glycosylated and GPI-anchored. The cleavage in the β -site, located in the octapeptide region, produces a N-terminal fragment (N2) and a C-terminal glycosylated and GPI-anchored fragment (C2). N1 and N2 are released in the extracellular milieu, while C1 and C2 can be attached to the cell surface or released.

When PrP is synthesised in vitro (in transfected cells) or in mouse brain, it has been reported that some of the molecules may assume a transmembrane orientation (Hegde et al., 1998; Holscher et al., 2001; Kim et al., 2001). These species, designated ^{Ntm}PrP and ^{Ctm}PrP , span the lipid bilayer once via a highly conserved hydrophobic region in the centre of the molecule, with either the N-terminus or C-terminus, respectively, on the extracytoplasmic side of the membrane. Because in ^{Ntm}PrP and ^{Ctm}PrP a different part of the protein is accessible to exogenous proteases, digestion of the two transmembrane forms yields two different fragments. One fragment, corresponding to ^{Ctm}PrP , is COOH-terminal derived and glycosylated, and the other (from ^{Ntm}PrP) is NH_2 -terminal derived and unglycosylated. ^{Ntm}PrP and ^{Ctm}PrP are generated in small amounts (<10% of the total) as part of the normal biosynthesis of wild-type PrP in the endoplasmic reticulum (Figure 1.4). However, mutations within or near the transmembrane domain, including the A117V and P105L mutations linked to GSS, as well several artificial mutations not seen in patients, increased the relative proportion of ^{Ctm}PrP to as much as 20-30% of the total (Hegde et al., 1998; Hegde et al., 1999; Stewart and Harris, 2001). Current evidence indicates that the membrane topology of PrP is determined by competition at the translocon between two conflicting topological determinants in the polypeptide chain: the signal sequence that directs translocation of the N-terminus of the polypeptide chain to produce secreted PrP (^{Sec}PrP) or ^{Ntm}PrP , and the central hydrophobic domain that directs translocation of the C-terminus across the membrane to produce ^{Ctm}PrP (Kim and Hegde, 2002; Kim et al., 2001; Stewart and Harris, 2003).

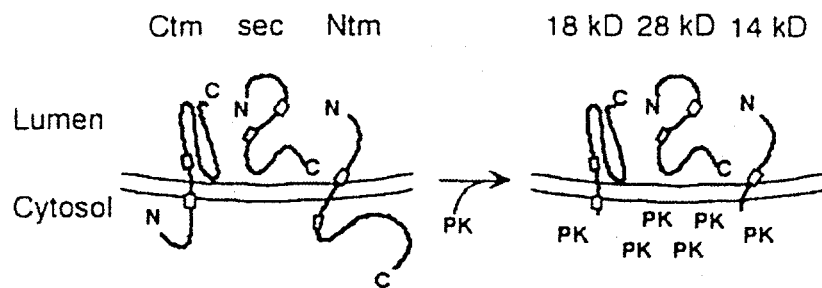


Figure 1.4: Schematic representation of ^{Ctm}PrP, ^{Ntm}PrP and secreted PrP. During translation a small percentage of PrP does not enter the ER but assumes a transmembrane orientation. In ^{Ctm}PrP and ^{Ntm}PrP the N-terminus and the C-terminus are exposed to proteases added to the cytoplasmatic side, while secreted PrP is fully protected. After PK digestion, three different forms are detected by Western blotting: a N-terminal fragment of 14 kD, a C-terminal fragment of 18 kD, corresponding respectively to the protected ^{Ntm}PrP and ^{Ctm}PrP, and a band of 28 kD corresponding to secreted PrP.

1.1.3 Function of PrP^C

The physiological function of the prion protein is unknown, although its localization on the cell surface would be consistent with roles in cell adhesion and recognition, ligand uptake or transmembrane signalling. Mice in which the PrP open reading frame of the gene has been disrupted display no gross development or anatomical defects (Bueler et al., 1992) but are reported in some studies to have electrophysiological and structural abnormalities in the hippocampus (Colling et al., 1996; Collinge et al., 1994; Curtis et al., 2003; Manson et al., 1994a) alteration in the circadian rhythm and changes in learning and memory (Nishida et al., 1997) and sleep pattern (Tobler et al., 1996). Other studies (Mabbott et al., 1997) revealed that proliferation of lymphocytes from PrP knock out mice by concanavalin A was significantly reduced to 50-80% that of wild-type PrP mice, suggesting a role for PrP in extra-neuronal tissues.

In contrast, Ngsk PrP^{0/0} mice (Sakaguchi et al., 1996), in which the ORF was deleted together with large portion of 5' intron and the 3' untranslated region, suffered extensive Purkinje cell loss with progressive ataxia. This phenotype was due to the ectopic expression of the PrP-like protein Doppel in the brains of these mice (Li et al., 2000a; Li et al., 2000b; Moore et al., 1999). Doppel is encoded by the *Prn-d* locus, which is 16 kb downstream of the PrP gene and is not normally expressed in the CNS. In Ngsk PrP^{0/0} mice, as a consequence of the ablation of the *Prn-p* locus, the acceptor splice site of the third *Prn-p* exon is lost, causing exon skipping and formation of chimeric transcripts that place *Prn-d* under the control of the *Prn-p* promoter. Doppel toxicity was abolished by the presence of single wild-type *Prn-p* allele suggesting a functional interaction between the two genes (Mastrangelo and

Westaway, 2001; Moore et al., 2001; Nishida et al., 1999; Rossi et al., 2001). In particular, the octapeptide repeats region was required to rescue the phenotype (Atarashi et al., 2003).

Although the normal, physiological role of PrP^C remains enigmatic, there is evidence that the protein binds copper and may also play a role in the trafficking of copper ions. Redox-active transition metal ions such as copper and iron have a dual significance in relation to neurodegeneration (Sayre et al., 1999). These metals are essential as cofactors for many enzymes, and their deficiency can lead to dysfunction in the CNS. Thus, the copper chelator cuprizone has long been recognized to cause a neuropathy in rodents that appears somewhat similar to scrapie (Carlton, 1969). On the other hand, elevated levels of these ions can also evoke disturbances characterized by oxidative stress and free-radical production. The amino-terminal section of PrP (spanning residues 51-71 of human PrP) contains a series of glycine and histidine-containing peptides repeats, and the interaction of this region with Cu²⁺ has been studied in some detail (Brown et al., 1997a; Stockel et al., 1998). Synthetic peptides from this region bind Cu²⁺ with a K_d in the micromolar range, thus suggesting that the interaction between copper and PrP is physiological. Various studies have explored different aspects and consequences of the binding of Cu²⁺: 1) Cu²⁺ has been shown to facilitate the return of protease resistance and infectivity after denaturation of PrP^{Sc} with guanidine (McKenzie et al., 1998); moreover, studies in vitro indicate that copper favours the conversion of PrP^C to a protease-resistant form that is distinct from PrP^{Sc} (Quaglio et al., 2001); 2) in transfected lines of N2a mouse neuroblastoma cells expressing mouse or chicken PrP, Cu²⁺ stimulates internalisation of PrP (Pauly and Harris, 1998; Perera and Hooper, 2001); 3) the content of copper in

membrane preparations from PrP null mice is about 20% of that in wild-type mice, and it has been argued that the neuronal Cu-Zn SOD (copper-zinc superoxide dismutase) is less active in PrP null mice and correspondingly higher in transgenic mice that over express PrP (Brown et al., 1997a; Brown et al., 1997b). However, other data indicate that PrP expression level does not influence the copper content of the brain, nor does it make any measurable contribution to dismutase activity (Waggoner et al., 2000). Moreover, when mice lacking the Sod 1 gene were crossed to PrP^{0/0} mice or with mice expressing different level of PrP, no influence of the PrP dosage was detected on the SOD activity (Hutter et al., 2003).

A second perspective on the possible roles of PrP^C at the cell surface has come from the study of PrP null mice expressing PrP molecules with amino-proximal deletions (Shmerling et al., 1998). In mice expressing PrP molecules in which aminoacids 32-80, 32-93 and 32-106 were deleted there was no phenotype, but animals with longer deletions, from 32-121 or 32-134, showed extensive death of neurons. Based on these observations, and given the localisation of PrP as a GPI-anchored proteins in rafts, it was proposed that PrP^C might elicit a cell survival signal upon binding to a putative receptor (or ligand), and that deleted forms of PrP could bind to the receptor but fail to supply this neurotrophic signal (Shmerling et al., 1998). Further to this, and in support of a neuroprotective role for PrP, it has been shown that neuronal cell cultures from PrP^{0/0} mice are more susceptible to oxidative stress (Brown et al., 1997b; White et al., 1999) and serum deprivation (Kuwahara et al., 1999). Moreover, *in situ* hybridisation analysis of human brains with cerebral ischemia or perinatal hypoxic ischemia revealed upregulation of PrP mRNA, suggesting a role for PrP in the neuroprotective cellular response to hypoxic injury. These

data have been confirmed in transgenic mice, being PrP^{0/0} mice more susceptible to hypoxic injury than wild-type mice (McLennan et al., 2004). Reintroduction of a single PrP allele, as in hemizygous mice, was only partially protective, suggesting a quantitative effect.

These findings have prompted a search for PrP-interacting partners and several molecules have been found to interact with PrP, including laminin receptor precursor (Rieger et al., 1997), and plasminogen (Fischer et al., 2000). However, none of these interactors have yet revealed a functional pathway in which PrP would be involved *in vivo*.

Recently, PrP has been shown to be involved in different signal transduction pathways related to cell survival. First, it was shown that antibodies that cross-linked cell surface PrP stimulated the activation of Fyn tyrosine kinase (known to be associated with cellular proliferation and survival) in murine 1C11 differentiated neuronal cells (Mouillet-Richard et al., 2000). Second, it has been demonstrated that PrP binds to Bcl-2 in the yeast two-hybrid system (Kurschner and Morgan, 1995), that transfection of PrP or Bcl-2 rescued the PrP null cells from apoptosis induced by serum deprivation (Kuwahara et al., 1999) and that PrP protected human primary neurons from Bax-mediated cell death (Bounhar et al., 2001; Roucou et al., 2003). This neuroprotective role was abolished in PrP constructs lacking the octapeptide repeats or carrying the D178N mutation (Roucou et al., 2003). The neuroprotection of PrP required mature PrP but not the GPI anchor, indicating that cell surface signaling may be not the only protective pathways of PrP. Third, a PrP^C binding peptide has been shown to prevent apoptosis, by activating cAMP protein kinase A (PKA) and Erk pathways (Chiarini et al., 2002). The PrP binding protein was identified as the stress-inducible protein 1 (STI1) which is present in the cytoplasm and at the

cell surface. Cell surface binding experiments showed that recombinant PrP^C binds to STI1 and co-immunoprecipitation assay suggest that the two proteins are associated *in vivo*. The authors proposed that the interaction could induce neuroprotective signals that rescued cell from apoptosis (Zanata et al., 2002). Whereas the Fyn and STI1 related transduction functions appear to occur through the cell surface GPI-anchored protein, the anti-Bax function could be mediated by cytosolic PrP or secreted PrP.

1.1.4 TSE strain variation

It is well established that TSE agents, like conventional micro-organism, exhibit strain variation. Numerous distinct TSE strains have been identified in mice by serially passaging scrapie (Bruce, 1993; Bruce and Dickinson, 1985), BSE (Bruce et al., 1994; Fraser et al., 1992) or CJD (Bruce et al., 1997; Collinge et al., 1996) from a range of sheep, goat, cattle and human sources. The methods used for TSE strain discrimination have traditionally been based on simple observation of disease characteristics. The most useful have been the length of the incubation period between infection and the appearance of clinical symptoms and the type and distribution of pathological changes that are seen in the brain of infected animals (Bruce et al., 1991; Dickinson and Meikle, 1971; Dickinson et al., 1968b; Fraser and Dickinson, 1973). TSEs strains have also found to differ in their clinical manifestation and their ease of transmission to new species. Prions exhibiting different biological properties were first recognized in goats with scrapie where different clinical phenotypes were manifested (Pattison and Millson, 1961). A link between PrP biochemical properties and strains was established with the finding that PrP^{Sc} associated with various murine scrapie strains differed in the relative proportion of PrP^{Sc}

glycoforms (Kascsak et al., 1991). More recently when transmissible mink encephalopathy (TME) was transmitted to hamsters, two different strains (called "hyper" and "drowsy" because of the differences in clinical phenotype), were identified (Bessen and Marsh, 1992). Purification and analysis of PrP^{Sc} from animals infected with hyper and drowsy agent revealed differences in biochemical properties: in particular, PrP from drowsy-infected brains was more soluble in detergents and more sensitive to proteinase-k (PK) digestion; moreover, the site of protease cleavage was different. To explain these differences, the authors suggested the existence of different conformations of PrP. Later, it was demonstrated that these strain-specific properties could be transmitted to PrP^C in a cell free-system, providing evidence for self-propagation of PrP^{Sc} (Bessen et al., 1995). Finally, spectroscopy studies demonstrated that PrP^{Sc} purified from hyper and drowsy did indeed show different conformation (Caughey et al., 1998). Different strains of TSE agent are associated with different glycoform ratios and different sizes of the PrP^{Sc} domain that is resistant to digestion by proteinase-K (Collinge et al., 1996; Parchi et al., 1996). Precisely how these strain-specific glycoform ratios are maintained is currently an open question. They may be the consequence of preferential conversion of a particular PrP^C glycoform into PrP^{Sc}. In this instance, it would be the strain-specific PrP^{Sc} that determines the final glycoform pattern (Somerville, 1999). Alternatively, if there are differences in the PK-sensitivities of the different glycoforms of PrP^{Sc}, degradation of the newly formed PrP^{Sc} in endolysosomal compartments could account for the different banding pattern seen in different strains (DeArmond et al., 1999). Alternatively, the infected host may play a role in the molecular aspects of TSE strain pathogenesis. Thus, differences in PrP^C glycosylation in different population of

neurons of the brain could target incoming infectivity to specific population of neurons. This could be modulated by the possible binding of some ligand playing a role in strain associated PrP-diversity. The complex interplay between PrP^{Sc} conformation, glycoform selection and ligand binding may cause PrP^{Sc} strains to target and to be most efficiently propagated within subsets of cells that can provide the preferred glycoform and ligand. This cellular targeting, as well as differences in susceptibility of target cells to the toxic effect of PrP^{Sc} may then establish strain-dependent pattern of pathological lesion and the consequent phenotype of disease (Bruce et al., 1989). The existence of different conformations of PrP and the hypothesis that each of them can target to different regions of the brain would therefore explain the large phenotypic variability described for human prion diseases (see section 1.4).

1.2 The prion hypothesis

Scrapie has been known as a naturally occurring disease for over 250 years in various parts of Europe (Parry, 1984). In 1936 Cuillé and Chelle demonstrated the transmissibility of the disease by experimental inoculation, but with a long incubation period. At that time, some type of virus was assumed to be the causative agent and Sigurdsson, because of the long incubation period, coined the term "slow virus" (Sigurdsson, 1954). Later, it was observed that the scrapie agent had a very low molecular weight, was resistant to procedures that inactivated or modify nucleic acids but was sensitive to treatments that denature proteins (Alper et al., 1967; Pattison and Jones, 1967). This evidence suggested that the infectious agent could be a protein in nature, lacking a nucleic acid component but it was not clear how it could replicate itself. Griffith

proposed three different mechanisms to explain the self replication of proteins: 1) the "infective" protein could be a transcriptional activator of the gene (normally silenced) coding for the protein itself; 2) the "infective" protein could be a conformationally different form of a cellular protein and was able to confer its conformation to the normal protein by physical interaction; 3) the "infective" protein could be able to stimulate the production of antibodies identical to the protein (Griffith, 1967).

Building on the second mechanism proposed by Griffith, Prusiner proposed the existence of a novel class of infectious agent that he named prions (proteinaceous infectious particle) (Prusiner, 1982). Attempts to purify the infectious agent from brains of hamsters infected with scrapie led to the discovery that the infectivity co-purified with a protein of 27-30 kDa, which was a protease-resistant fragment of a 33-35 kDa precursor protein. This proteinase resistant form of the protein, which was named PrP^{Sc} , for scrapie isoform of the prion protein, was found to be a conformationally altered isoform of a cellular protein, called PrP^{C} , for cellular prion protein (Oesch et al., 1985). According to the prion hypothesis, PrP^{C} is converted into PrP^{Sc} by a post-translational process that appears to involve a conformational change, with transition of α -helices into β -sheets (Figure 1.5) (Caughey et al., 1991b). Stochastic fluctuations in the structure of PrP^{C} would generate a partially unfolded monomer (PrP^*) that is an intermediate in the formation of PrP^{Sc} . PrP^* can revert to PrP^{C} , be degraded or form PrP^{Sc} . Normally the concentration of PrP^* would be low and PrP^{Sc} would be formed in insignificant amount (Cohen et al., 1994). In the case of infectious prion diseases, exogenous prions containing PrP^{Sc} would act as a template to promote the conversion of PrP^* into PrP^{Sc} (Cohen et al., 1994). In the case of inherited prion diseases, PrP mutations

would destabilize PrP and promote its conversion into PrP* (Cohen et al., 1994). Indeed, the majority of point mutations that segregate with inherited diseases fall within or near the four putative α -helices (Hsiao et al., 1989). Finally, the sporadic forms may result from the rare occasions in which there is sufficient accumulation of PrP* to produce PrP^{Sc} (Cohen et al., 1994). Based on experimental and computational results it has now been suggested that the fundamental unit of PrP^{Sc} structure is a trimer of parallel left-handed β -helices; moreover, trimers could assemble by hydrogen bonds determining protein aggregation (Govaerts et al., 2004; Wille et al., 2002) (Figure 1.5).

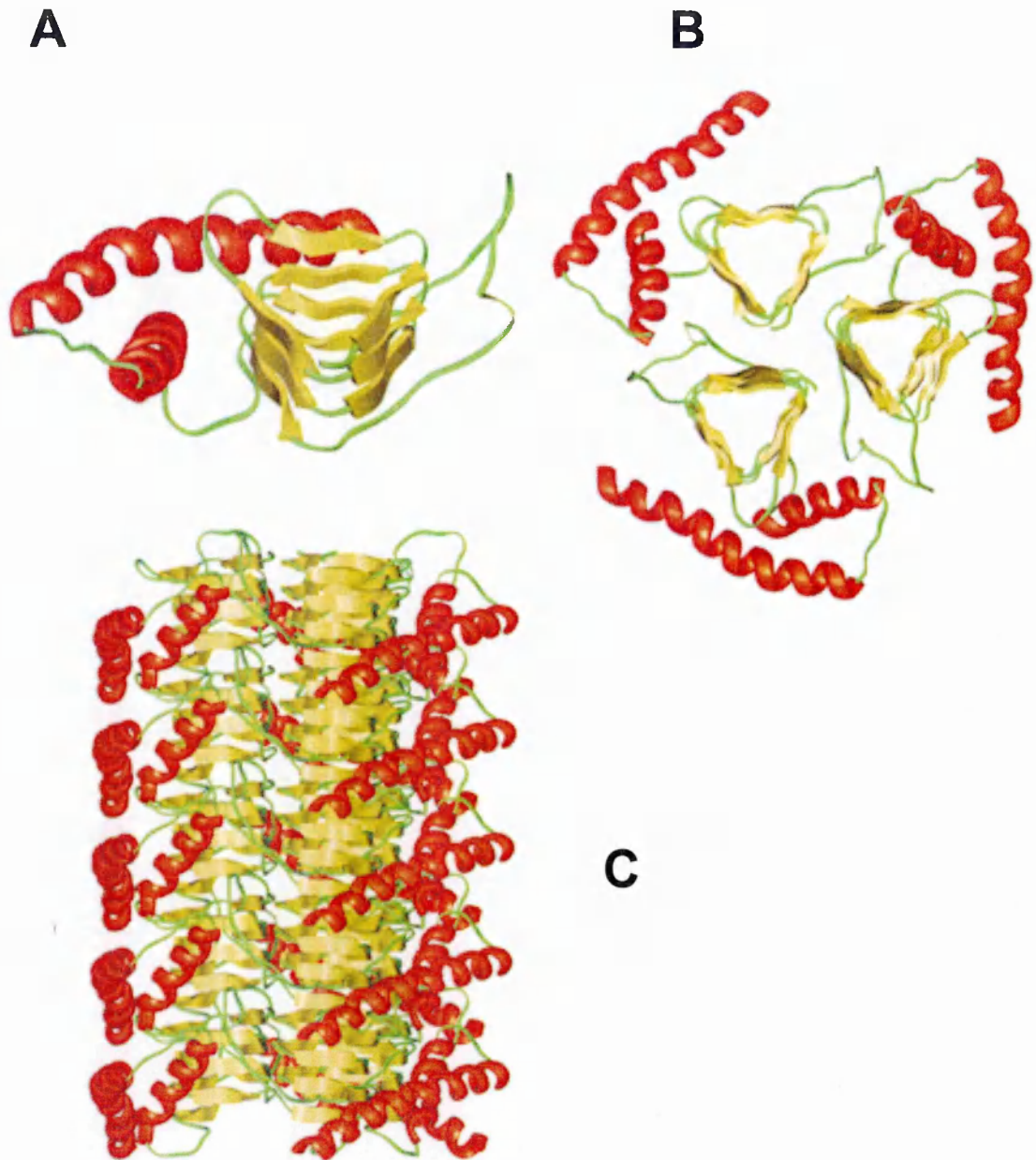


Figure 1.5: Modelling of PrP^{Sc} formation.

(A) A model of the monomer of mouse PrP^{Sc}: the main conformational change occurs between residues 89 and 170 while the two C-terminal α -helices of PrP^C are retained. (B) Trimeric model of PrP^{Sc} built by superimposing three monomeric models (C) A model for PrP^{Sc} fiber constructed by assembling five trimeric disc.

PrP^{Sc} differs from PrP^C in several physicochemical properties: it is partially resistant to PK digestion and forms aggregates, determining its insolubility in non-denaturing detergents. The tendency to aggregate also determines the formation of fibrillar structures called prion rods that show the typical fluorescent birefringence of amyloid when stained with Congo Red (Prusiner et al., 1983). Probably because of the resistance to protease, PrP^{Sc} accumulates in the brains of affected animals and humans, where it is thought to propagate itself by impressing its abnormal conformation on PrP^C, generating additional molecules of PrP^{Sc}.

Two mechanisms have been proposed for PrP^{Sc} formation (Figure 1.6). The heterodimer model posits that PrP^{Sc} exists in a stable monomeric state that can bind PrP^C forming an heterodimer, and catalyses a conformational change in PrP^C to form an homodimer of PrP^{Sc} (Bolton and Bendheim, 1988; Griffith, 1967). The two PrP^{Sc} molecules then separate to give two PrP^{Sc} monomers. The fundamental underlying postulates of this model are that PrP^{Sc} is more stable thermodynamically than PrP^C, conversion of PrP^C to PrP^{Sc} is rare unless catalysed by pre-existing PrP^{Sc} and that the PrP^{Sc} homodimer tends to dissociate. In the nucleated polymerisation model (Gajdusek, 1988; Jarrett and Lansbury, 1993), oligomerization of PrP is necessary to stabilize PrP^{Sc} sufficiently to allow its accumulation to biologically relevant levels. Spontaneous formation of seeds of PrP^{Sc} is rare because of the weakness of monovalent interactions between PrP^C molecules and the oligomer. However, once formed, oligomeric or polymeric seeds are stabilized by multivalent interactions. In familial and sporadic human TSEs, formation of an initial template (or seed) might be a spontaneous and stochastic event that can be potentiated by specific mutations (Caughey et al., 1995; Jarrett and Lansbury, 1993). In TSEs

of infectious origin transmission might be explained by acquisition of preformed PrP^{Sc} template or seed.

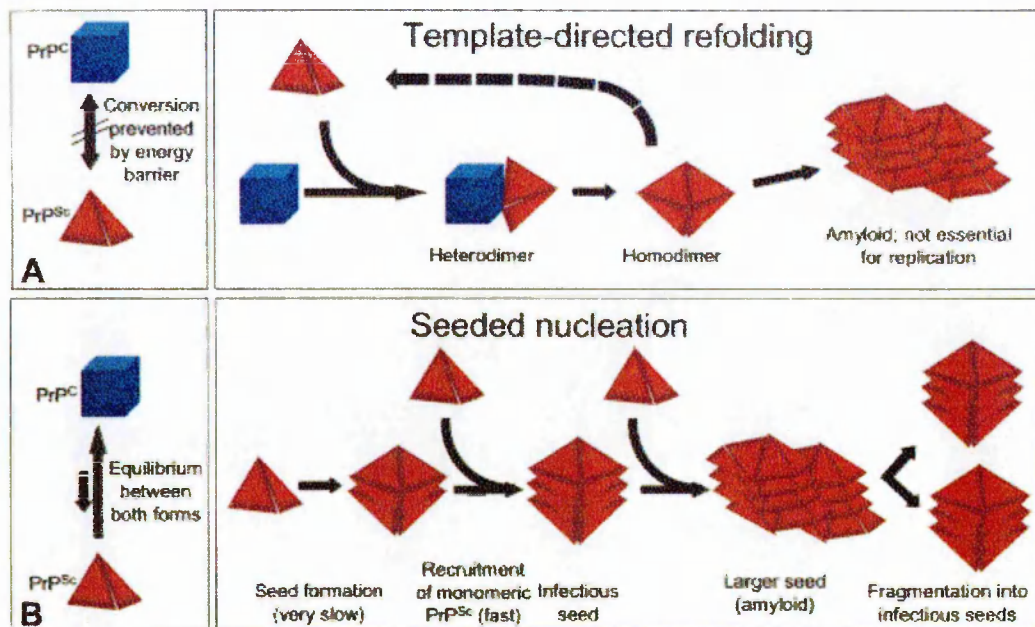


Figure 1.6: Mechanistic models for formation of PrP^{Sc} from PrP^C
(Aguzzi and Polymenidou, 2004)

Support for the concept that PrP^{C} is converted to PrP^{Sc} in the presence of pre-existing PrP^{Sc} has been derived from "conversion assay" in cell-free models. In these experiment $[^{35}\text{S}]$ methionine-labeled PrP^{C} was converted into proteinase-K resistant ^{35}S -PrP in the presence of purified PrP^{Sc} (Kocisko et al., 1994). This converting activity was dependent on the initial concentration of PrP^{Sc} and was associated only with aggregates (Caughey et al., 1995). The cell-free conversion of PrP^{C} to PrP^{Sc} was also demonstrated *in situ*, by incubating purified $[^{35}\text{S}]$ methionine-labeled PrP^{C} with brain slices of TSE-infected animals. In particular, proteinase-K resistant ^{35}S -PrP was found exactly at the sites of endogenous PrP^{Sc} deposits (Bessen et al., 1997). The cell-free conversion reaction studies validate the prion hypothesis, indicate that PrP conversion activity is associated with PrP^{Sc} aggregates and therefore support the nucleation-dependent protein polymerization hypothesis.

A strong prediction of the prion hypothesis is that expression of PrP by the host is necessary for the propagation of the infectious agent and the development of the disease. In accordance with this prediction, it has been convincingly shown that *Prn-p*^{0/0} mice, in which the prion protein gene has been inactivated through homologous recombination, are resistant to prion infection and do not sustain replication of the infectious agent (Bueler et al., 1993; Caughey et al., 1995). *Prn-p*^{0/0} mice remained completely healthy even when transgenic embryonic neuronal tissue overexpressing PrP was transplanted into their brain and infected with scrapie prions (Brandner et al., 1996a). The graft accumulated high level of PrP^{Sc} and infectivity that migrated into the host brain, but only the graft developed the typical histopathological changes of scrapie, with no replication of PrP^{Sc} in the null tissue. Recently it has been demonstrated

(Mallucci et al., 2003) that depleting endogenous neuronal PrP^C in conditional PrP knockout mice with established neuroinvasive prion infection reversed early spongiform change and prevented neuronal loss and progression to clinical disease. This occurred despite the accumulation of extra-neuronal PrP^{Sc} to levels seen in terminally ill wild-type mice. These last results, together with the neurografts experiments, clearly demonstrate that the continuous PrP expression in neurons is necessary for the onset and progression of the disease.

There is also compelling evidence that the primary sequence of PrP encoded by the host is a major determinant of the species-specificity, incubation time, and neuropathological characteristics of prion transmission (Bessen et al., 1995; Bessen and Marsh, 1992). For example transgenic mice harboring hamster PrP were susceptible to hamster prions while non transgenic (Tg) mice did not (Scott et al., 1989). Tg mice expressing both mouse and hamster PrP could be infected with prions from both species but exhibited typical neuropathological changes characteristics of the species inoculated (Prusiner et al., 1990). These and other lines of evidence prove that PrP plays a key role in the disease process, and it has become increasingly difficult to explain all the existing data by a viral theory of pathogenesis. What some have considered to be a significant argument against the prion hypothesis, the existence of prion strains that produce different incubation times and neuropathological profiles in a single host, can now be explained by evidence that each strain may represent a distinct, self propagating conformation of PrP^{Sc} (see section 1.1.4).

The recent finding that recombinant mouse PrP(89-231) polymerised into amyloid fibrils produces neurological dysfunction when injected intracerebrally into transgenic mice expressing PrP(89-231) and that the disease is

transmissible to non-transgenic mice in serial passages, constitute the most compelling demonstration of the validity of the prion hypothesis (Legname et al., 2004).

1.3 Yeast and fungal prions

The prion hypothesis has now been extended to explain phenomena involving other proteins. Using genetic criteria, the non-Mendelian yeast elements [URE3] and [PSI⁺] were identified as prions of the Ure2 and Sup35 proteins, respectively (Wickner, 1994) (Figure 1.7). (Note: the convention here is that capital letters signify dominance, brackets signify nonchromosomal inheritance). [URE3] results in a deregulation of nitrogen catabolite repression and is derived from an aggregated state of the Ure2 protein. Yeast cells presented with a rich nitrogen source, such as ammonia, shut off production of enzymes necessary for the utilization of poor nitrogen sources. A poor nitrogen source, such as proline, does not shut off utilization of other poor sources. This phenomenon is called "nitrogen catabolite" (Cooper et al., 1992). When ammonia is supplied as a nitrogen source, ureidosuccinate (USA) is not normally taken up, while it is taken up when a poor nitrogen source like proline is supplied. Mutants able to take up USA in the presence of ammonia defined the chromosomal ure1 and ure2 genes and the nonchromosomal genetic element [URE3] (Lacroute, 1971) (Figure 1.7A). [PSI⁺] enhances the suppression of nonsense codons and is derived from the "prion state" of Sup35 protein. Sup35 is a subunit of the termination factor that recognizes the termination codon and cleaves the completed peptide from the final tRNA, thereby terminating translation. Mutations in the SUP35 gene or conversion of

Sup35 protein to a prion impairs the normal termination process (Figure 1.7B)
(Cox, 1965).

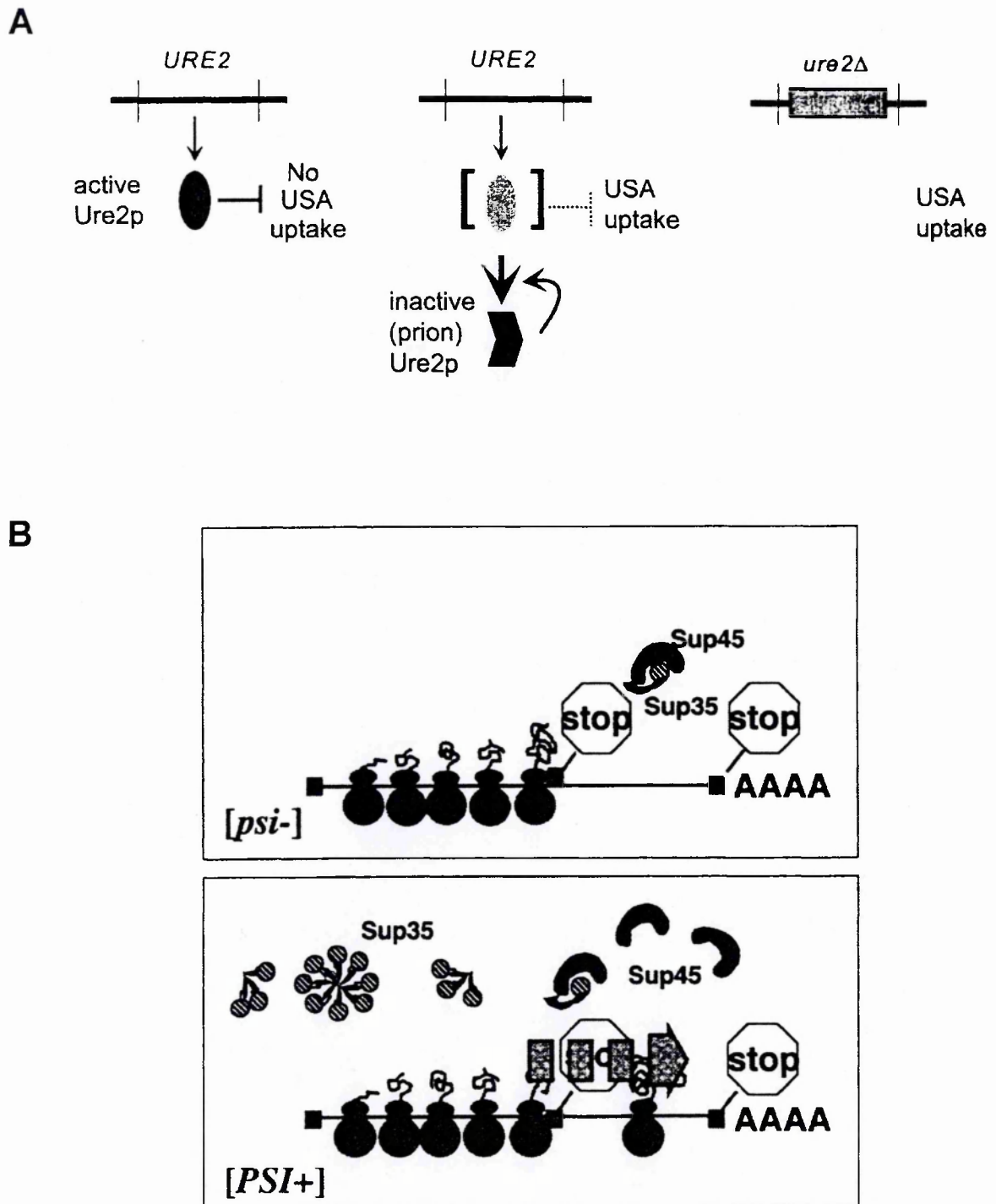


Figure 1.7: Phenotype of yeast prions [URE3] and [PSI+].

(A) In a rich nitrogen source USA is not taken up by yeast cells. However, when Ure2p protein acquires the prion state or when the corresponding gene is ablated, USA is taken up by the cells (Wickner et al., 2001).

(B) In *[psi-]* cells (top), Sup35 is soluble and binds to Sup45. The Sup35/Sup45 complex promotes translation termination at nonsense codons. In *[PSI+]* cells, Sup35 forms aggregates and can not complex to Sup45. Consequently, ribosomes are able to read through nonsense codons (Serio and Lindquist, 2001).

Among the earliest and strongest genetic indications that [URE3] and [PSI⁺] were "prion" forms was the finding that their *de novo* appearance was efficiently induced by overexpression of the URE2 and SUP35 genes, respectively. These results were interpreted as providing evidence for the prion model since an excess of the protein should increase the probability that the prion form of the protein would appear and start a self-amplifying chain reaction, establishing the prion state. In further support of the "protein only" hypothesis, the *de novo* induction of the [URE3] and [PSI⁺] prions was directly shown to be caused by an excess of the protein. This finding established that the proteins alone were sufficient to cause the appearance of [URE3] or [PSI⁺] (Wickner, 1994).

Unlike PrP^{Sc} however, yeast prions are not pathogenic. Rather, they produce changes in the phenotype that mimic conventional loss-of-function mutations. The patterns of their inheritance, however, are very different. The loss-of-function phenotypes caused by yeast prions are dominant, rather than recessive. Moreover, they are inherited in a non-Mendelian fashion. When a haploid [URE3] or [PSI⁺] strain (producing the prion form) is mated to haploid [ure3⁻] or [psi⁻] (producing the non-prion form), the resulting diploid has a suppression phenotype, that is respectively, [URE3] or [PSI⁺]. On sporulation, none of the progeny are [ure3⁻] or [psi⁻], as expected for a nuclear determinant. Instead, [URE3] or [PSI⁺] are transmitted to all progeny (Cox, 1965; Lacroute, 1971).

Ure2 and Sup35 are normally soluble proteins, but they form protease K-resistant aggregates upon conversion to the prion state *in vivo* (Masison and Wickner, 1995; Patino et al., 1996). Aggregates isolated from extracts of [PSI⁺] or [URE3⁺] cells seed the rapid conversion of the purified corresponding

proteins into amyloid fibers (Paushkin et al., 1997). The induction of the prion state could also be obtained when only a portion of the proteins, called the prion domain, was overexpressed (Masison and Wickner, 1995). This domain, responsible for the prion behaviour, shows the following characteristics: 1) it is extremely rich in the polar residues glutamine and asparagines (DePace et al., 1998); 2) it has no definite secondary structure (Thual et al., 1999); 3) it is required for prion propagation, as shown by studies with partially deleted proteins (Masison and Wickner, 1995); 4) it can exist as soluble species or ordered self-perpetuating aggregates (Taylor et al., 1999).

The [Het-s] prion from the filamentous fungus *Podospora* has also been proposed as a prion-like protein. It is believed to mediate the barrage reaction of incompatibility after cell fusion, by promoting programmed cell death when incompatible cells attempt to fuse (Coustou et al., 1997). Recently, it has been suggested that a neuronal member of the CPEB family (cytoplasmic polyadenylation element binding protein), which regulates mRNA translation in the sea-slug *Aplysia*, also possesses prion-like properties (Si et al., 2003a; Si et al., 2003b). Like the [Het-s] prion, it is the dominant, self-perpetuating prion-like form of CPEB that has the greatest capacity to stimulate translation.

These findings demonstrate that the conformational change is a common mechanism for other proteins. However, while abnormal conformations of PrP are associated to pathogenicity, prions in yeast and fungi determine a change in the phenotype. The recent finding that other neuronal proteins besides PrP (Si et al., 2003a; Si et al., 2003b) might be able to propagate their conformation indicates that this molecular transformation could serve important physiological functions also in the central nervous system of mammals.

1.4 Human prion diseases

One of the many challenging features of the prion diseases is the prominent heterogeneity. While the pathology of other diseases of the central nervous system, such Alzheimer's disease, Huntington's corea and Parkinson's disease are rather homogeneous, prion diseases include a wide spectrum of histopathological phenotypes (Gambetti et al., 2003a). The heterogeneity reflects several factors. Firstly, prion diseases are unique among the diseases described above, because some forms have clearly been shown to be infectious. Second, depending on the origin of the exogenous infectious prion, the form of the disease acquired by infection may display phenotypes that are quite different. This is the case of iatrogenic CJD and vCJD that display different pathological features (Brown et al., 2000). Third, there are a large number of mutations that are associated with quite different disease phenotypes (Ghetti et al., 1995; Parchi et al., 1999). Fourth and finally, the sporadic forms of CJD themselves have an unusual degree of phenotypic heterogeneity (Parchi et al., 1996) (Table I.IV).

Table I.IV: Clinical and histopathological differences in human prion diseases

Disease	Age of onset /incubation period	Clinical features	Neuropathology
Sporadic CJD (Gambetti et al., 2003a)	45-75 years	Dementia, myoclonus, variable cerebellar dysfunction	Spongiosis, astrogliosis
Iatrogenic CJD (Brown et al., 1992)	1.5-30 years	Dementia, myoclonus, variable cerebellar dysfunction	Spongiosis, astrogliosis
vCJD (Will, 2003)	14-70 years (average=28)	Behavioural and psychiatric disturbances, cerebellar ataxia	Florid amyloid plaques
Familial CJD (Gambetti et al., 2003a)	20-85 years	Dementia, myoclonus, variable cerebellar dysfunction	Spongiosis, astrogliosis
GSS (Ghetti et al., 1995)	20-60 years	Cerebellar dysfunction, pyramidal motor dysfunction, intellectual deterioration	Amyloid plaques
FFI (Gambetti et al., 1995)	25-61	Insomnia, motor dysfunction	Thalamic involvement
Kuru (Goldfarb, 2002)	30 years (average)	Cerebellar ataxia, pyramidal motor dysfunction	Amyloid plaques

Recent understanding of the molecular mechanisms that play a central role in the pathogenesis of prion diseases has provided insight into the modalities of the phenotypic heterogeneity of these diseases. Codon 129 of the PrP gene is the site of a common methionine(M)/valine(V) polymorphism. In the Caucasian population, 36% of individuals are homozygous MM, 52% are heterozygous MV and 12% are homozygous VV (Collinge et al., 1991). It was observed that the phenotype of prion diseases, whether sporadic, familial and acquired, was often different depending on the genotype at codon 129. This codon plays a two-fold role in modulating the disease outcome. On the mutant allele, it determines the basic features of the disease phenotype, as illustrated by mutation at codon D178N (mutation in the genome resulting in the aspartate residue at position 178 being replaced by asparagine), which can be linked to CJD or FFI based on the residue specified at codon 129 of the mutant allele. In particular, D178N/129Val is always associated with a familial form of CJD, while D178N/129Met is associated with FFI. On the normal allele, codon 129 may modulate the severity of the phenotype (Collinge et al., 1991; Palmer et al., 1991). To explain phenotypic variability, it has been hypothesised that distinct *PRNP* variants may result in PrP isoforms having different properties and topographic distribution that determine the phenotypic expression of disease. In this regard, it has been found that PrP extracted from FFI and CJD brains differ in their degree of glycosylation and size of protease-resistant core (Monari et al., 1994).

As mentioned above (section 1.1.1), four diseases affect humans: Kuru, Creutzfeldt-Jacob Disease (CJD), Gerstmann-Sträussler-Scheinker Syndrome (GSS) and fatal familial insomnia (FFI) (Table I.I and I.IV). About 85% of human prion disease occurs sporadically as CJD at an approximate rate of 1 case for

million of population per year that is uniform around whole world (Collinge, 1997).

Kuru is geographically restricted to the Opaka area of the highlands of Papua-New Guinea (Gajdusek and Zigas, 1957). Over 2700 cases of Kuru have been documented since 1957 in a total population of 36,000 people. The possibility of transmission through endocannibalism was raised by anthropological enquiry (Gajdusek, 1977) and the decline in the epidemic following the cessation of this ritual in the tribes is consistent with the mechanism of transmission (Goldfarb, 2002; Will, 2003). Women and children consumed diseased relatives as a mark of respect, leading to the spread of the infection. Clinically, cases of Kuru presented with a pure cerebellar syndrome and the total illness duration ranged from 6-36 months (Gajdusek and Zigas, 1957; Zigas and Gajdusek, 1957). Genetic studies indicate that homozygosity at codon 129 (particularly for methionine) was associated with an earlier age of onset and a shorter duration of illness than was heterozygosity, but other clinical characteristics were similar for all genotypes (Cervenakova et al., 1998).

CJD occurs primarily as a sporadic disorder and rare cases have resulted from iatrogenic transmission. The two most frequent causes of iatrogenic CJD have arisen following implantation of dura mater grafts and treatment with growth hormone or gonadotropin prepared from human pituitary glands from individuals incubating the disease. The transmission of CJD by cadaveric dura mater grafts was first recognized in 1987 and there have now been at least 136 cases world-wide (Will, 2003). About 30,000 children had been treated with pituitary-derived growth hormone (hGH) by 1985, when the occurrence of three CJD in two hGH recipients provided strong evidence of transmission via hGH. hGH production required the pooling of many thousands

of glands and it is presumed that contamination of the hormone preparation occurred when pituitary glands derived from patients who had died from CJD or incubating the disease were included in the production process (Will, 2003). Less frequent occurrences of iatrogenic CJD have resulted from corneal transplantation, contaminated electroencephalographic (EEG) electrode implantation and surgical operations using contaminated instruments (Brown et al., 1992).

Approximately 15% of cases of CJD are inherited in a dominant fashion and linked to insertional or point mutation in the *PRNP* gene. Insertional mutations consist of 1, 2, 4, 5, 6, 7, 8 and 9 octapeptide repeat insertions between codons 51 and 91 of PrP. Several point mutations determine a CJD phenotype, the most common of which are the substitution aspartate>asparagine at codon 178 (coupled with valine at codon 129), glutamic acid>lysine at codon 200, valine>isoleucine at codon 210. Other cases of familial CJD are associated with rare mutations, i.e. the substitution valine>isoleucine at codon 180, threonine>alanine at codon 183 and methionine>arginine at codon 232 (Gambetti et al., 2003b) (Figure 1.8). The term Creutzfeldt-Jakob was introduced in 1922 following the reports of two German physicians, Hans Gerals Creutzfeldt and Alfons Maria Jakob of six cases with a novel neurodegenerative disease (Creutzfeldt, 1920 ; Jakob, 1921). From the beginning CJD became controversial as questions were soon raised concerning the homogeneity of the cases reported by the two physicians. The heterogeneity was further underlined by the number of subtypes identified by different authors. The most common subtype is recognized clinically by the occurrence of progressive dementia associated with myoclonus, pyramidal, extrapyramidal and cerebellar signs. The neuropathological hallmarks of the

disease are neuronal loss, astrogliosis and spongiform changes. Lesions were observed to affect variably either the entire cerebral cortex or only certain layers selectively, and variability extends to basal ganglia and cerebellum (Gambetti et al., 2003a; Ironside, 1996).

In variant CJD (vCJD) the early clinical course is dominated by psychiatric symptoms, although a minority have neurological symptoms. After about 6 months, there are neurological signs, including ataxia, cognitive impairment and involuntary movements, which may be dystonic, choriform or myoclonic. Neuropathological analysis shows florid plaques extensively distributed in the cerebrum and cerebellum (Will, 2003)

GSS is a genetic form of prion disease that segregates with the following point mutations in *PRNP* gene (Ghetti et al., 1995): 1) proline>leucine at codon 102, 2) proline>leucine at codon 105, 3) alanine>valine at codon 117, 4) tyrosine>stop at codon 145, 5) phenylalanine>serine at codon 198, 6) arginine>glutamine at codon 217 (Figure 1.8). Although insert mutations are commonly found in CJD, to date GSS appears linked only to an eight octapeptide repeat expansion (Goldfarb et al., 1992). Clinical presentations of GSS are ataxia, extrapyramidal syndrome, spastic paraparesis and dementia. The pathological hallmark of the disease is the deposition of PrP amyloid in the central nervous system (Ghetti et al., 1995).

FFI is predominantly linked to the substitution aspartate>asparagine at codon 178 (Medori et al., 1992) with a methionine at codon 129 although recently a sporadic form of the disease has also been recognized (Montagna et al., 2003) (Figure 1.8). The disease is characterized clinically by untreatable insomnia, endocrine abnormalities and motor dysfunction. The neuropathological signature is a selective atrophy of the anterior ventral and

mediodorsal thalamic nuclei in which more than 50% of the neurons are lost.

This loss is associated with reactive astrogliosis (Manetto et al., 1992).

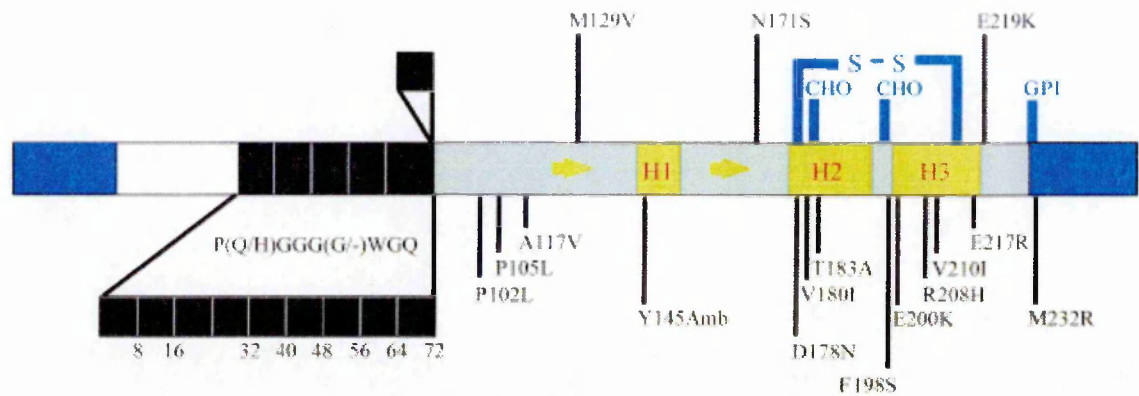


Figure 1.8: Structural features and pathogenic mutations of prion protein. Pathogenic mutations associated with human prion diseases are shown below the PRNP coding sequence. These consist of expansions of the octarepeat region (shown in dark) and point mutations causing aminoacids substitutions. Polymorphisms occurring in the human population are shown above the PRNP coding sequence. The three α -helices (H1, H2 and H3) and the two short β -sheets (yellow arrows) identified by NMR are represented. Protease resistant core of PrP^{Sc} is shown in gray (Telling, 2000).

1.5 Experimental approaches to prion diseases

1.5.1 Cell-free models

The conversion of PrP^C to neuropathological forms is central to the pathogenesis of TSEs. Although the mechanism of conversion from PrP^C to PrP^{Sc} and full neuropathological implications of PrP^{Sc} deposition *in vivo* remains uncertain, studies of PrP conversion in cell-free models have provided important insights into the circumstances in which PrP can adopt a PrP^{Sc}-like protease resistant state upon interaction with PrP^{Sc}. Thus, when incubated with PrP^{Sc} the PrP^C tends to bind to PrP^{Sc} and then slowly convert to a protease resistant state. This reaction has been shown to occur under a variety of conditions. The simplest contained mixture of purified [³⁵S]methionine-labeled PrP^C and purified PrP^{Sc} (Kocisko et al., 1994). The conversion reaction could be stimulated by detergent, chaperone proteins (DebBurman et al., 1997; Kocisko et al., 1994; Raymond et al., 1997), sulphate glycans and elevated temperature (Wong et al., 2001). Converting activity was associated with PrP^{Sc} polymers but not with soluble, monomeric PrP (Caughey et al., 1995). Kinetic analyses provided evidence that the conversion process could be separated into two stages, first the binding of PrP^C to PrP^{Sc} and then a slower conversion of the bound PrP^C to PrP^{Sc} (Bessen et al., 1997; Horiuchi and Caughey, 1999). Direct contact between PrP^C and PrP^{Sc} was found to be necessary, as studies of the conversion reaction between GPI-anchored, membrane bound PrP isoforms revealed that conversion was not efficient when PrP^C and PrP^{Sc} were attached to separate membrane vesicles. Conversion of PrP^C was only detected when vesicle were fused or PrP^C was detached from its GPI anchor and ceased to be

membrane bound (Baron et al., 2002). The conversion reaction has also been performed in scrapie infected brain slices (Bessen et al., 1997) and cellular extracts (Saborio et al., 1999).

In most of the above studies, the yield of PrP^{Sc} conversion was low; for this reason, in order to amplify PrP^{Sc} substantially *in vitro*, the “protein misfolding cyclic amplification” was developed (PMCA) (Saborio et al., 2001). In this system, detergent extracts of TSE-infected animals were mixed with vast excesses of similar extracts of PrP^C-containing normal brain tissue and subjected to repeated cycles of sonication and incubation. More than 30-fold increases in the amount of PrP resistant to PK digestion over that provided in the infected brain were reported. This result suggested that this procedure might be exploited to enhance the detection of PrP^{Sc} in TSE diagnostic tests. In addition, because the reported yield was much higher than the yields observed previously between purified proteins, it was suggested that unidentified auxiliary factors provided in the crude normal brain homogenate might be important in the PrP^C-PrP^{Sc} conversion (Saborio et al., 2001). One of these factors has been identified as an RNA species, since addition of mammalian mRNA stimulated amplification and treatment with RNase diminished the yield of the reaction (Deleault et al., 2003). To date, protease resistant PrP produced *in vitro* has lacked detectable infectivity (Bieschke et al., 2004; Hill et al., 1999), demonstrating a distinction between protease resistance and infectivity.

The cell-free conversion model has also been used to study the mechanisms related to the existence of strains (as described in Section 1.1.4) and to the species barrier. A species barrier in the TSEs is defined as the resistance to TSE disease by one host species after infection with the TSE agent of another species (this argument will be discussed in more detail in

Section 1.5.3.1). The fact that PrP^{Sc} binds PrP^C (DeBurman et al., 1997; Horiuchi and Caughey, 1999) suggests that the PrP aminoacid sequence may be involved in the conversion process. Many "heterologous" conversion reactions have been performed using PrP^C of one species (or sequence) and PrP^{Sc} of another. Profound sequence specificity was observed (Bossers et al., 1997; Kocisko et al., 1995; Raymond et al., 1997); in fact, in comparing reactions driven by a given PrP^{Sc}, 5 to 50-fold stronger conversion efficiencies can occur with PrP^C molecules from highly susceptible animals versus clearly resistant species/genotypes. It seems that the log of the relative intracerebral transmission titre might be roughly proportional to the relative cell-free conversion efficiency (Raymond et al., 2000).

Cell-free conversion assays have also been used to gauge the relative susceptibilities of various hosts to TSE agents from different source species. For instance, little is known about the transmissibility of chronic wasting disease of deer to non-cervid species (Miller et al., 2000). In cell-free conversion reactions, PrP^{Sc} purified from sick cervids readily induced the conversion of cervid PrP^C to the protease-resistant state (Raymond et al., 2000). In contrast, cervid PrP^{Sc}-induced conversion of human, bovine and ovine PrP^C was 14-100 fold, 5-12 fold and 2-3 fold less efficient, respectively, than the most convertible cervid PrP^C (Raymond et al., 2000).

Studies of the species specificity of conversion reaction have revealed that, in some interspecies combinations, the binding of PrP^C to heterologous PrP^{Sc} can occur more efficiently than the subsequent conversion to PrP^{Sc} (Horiuchi et al., 2000). Thus, the species specificity of PrP^{Sc} formation may be determined more by the conversion step than the initial binding step.

1.5.2 Cellular models

During the past two decades, considerable efforts have been made to establish culture models of genetic and infectious forms of prion diseases and especially cellular cultures supporting prion replication. These models present obvious advantages including: 1) the ability to analyse, at the molecular and cellular levels, the biological properties of PrP^C and PrP^{Sc}; 2) the possibility of determining the nature of the infectious agent and the factors determining its propagation; 3) the screening of drugs with potential therapeutic values; 4) the determination of biological markers of the infection with potential diagnostic and physiological interest.

1.5.2.1 Scrapie infected cells

The approach most frequently used to propagate prions *ex vivo* has been to incubate cell cultures in medium containing infectious fractions and to monitor for the replication of PrP^{Sc} (Butler et al., 1988; Race et al., 1988; Race et al., 1987; Taraboulos et al., 1990b). Following the inoculation of the culture with whole brain homogenates of infected animals, or with purified PrP^{Sc}, cells are generally washed extensively and serially passaged several times. The infected cultures continuously produce authentic PrP^{Sc} that can induce prion disease in laboratory animals (Race et al., 1987).

Several techniques currently exist to detect the presence of PrP^{Sc} in infected cells. The most common is proteinase-K resistance assay of cell lysates, followed by SDS-PAGE separation and Western blot analysis using anti-PrP antibodies. Protease resistance is manifested by production of an N-terminally truncated core of 27 to 30 kDa. Moreover, using antibodies that

selectively recognize PrP^{Sc}, it is possible to detect this form in an ELISA (enzyme-linked immunosorbent assay) assay. This technique is based on the previous finding that some antibodies raised against PrP^{Sc} do not recognize PrP^C (Korth et al., 1997; Paramithiotis et al., 2003). The explanation for this is that PrP^C and PrP^{Sc} differ in conformation and the epitope recognized by an antibody specific for PrP^{Sc} can be masked in PrP^C and *vice versa*. Recently other techniques have been established, such as cell blot (Bosque and Prusiner, 2000) and slot blot (Winklhofer et al., 2001). In the cell blot, cells are grown on a coverslip and subsequently transferred on a nitrocellulose membrane; in slot blots, cell lysates are filtered through a nitrocellulose membrane. In both cases PrP^{Sc} is revealed, after PK digestion, with anti-PrP antibodies.

Only some neuronally-derived cell lines appear to be susceptible to infection with scrapie prions *in vitro*. These have included N2a mouse neuroblastoma cells (Butler et al., 1988; Race et al., 1987), PC12 rat pheochromocytoma cells (Rubenstein et al., 1984) and T-antigen-immortalized hypothalamic neurons (GT1) (Schatzl et al., 1997). Several authors have tried to derive infected cultures directly from infected animals but only one group has succeeded, producing the SMB cell line (Clarke and Haig, 1970a; Clarke and Haig, 1970b). In some cases scrapie infected N2a display no obvious cytolytic changes (Butler et al., 1988) but in some other cases alterations were reported, such as increased chatecolamine and decreased free serotonin and noradrenalin levels (Markovits et al., 1983), or alterations in the bradykinin-mediated responses and in membrane fluidity (Kristensson et al., 1993; Wong et al., 1996). In PC12 cells differentiated with nerve growth factor (NGF), the infection was accompanied by decreases in the activity of enzymes involved in

the cholinergic neurotransmitter pathway (Rubenstein et al., 1991). Finally, a recent study demonstrated that prion infection impaired the cellular response to oxidative stress in GT1 cells (Milhavet et al., 2000). Unfortunately, it is not clear whether the changes described were necessary due to infection by the scrapie agent rather than to clonal differences or other factors present in the inoculum. Recently it has been demonstrated that brain-derived primary cells maintained in cultures can enter an infected state after exposure to scrapie agent (Cronier et al., 2004). In these experiments both neurons and astrocytes sustained prion propagation, leading to progressive neuronal loss.

Prion-infected cultures represent a useful model to understand the cellular and molecular events leading to the formation of PrP^{Sc}. Traditional immunofluorescence methods detected PrP^C on the cell surface of cultured cells (Stahl et al., 1987). However, when applied to scrapie-infected cells these methods failed to yield an additional, scrapie specific signal. The discovery that denaturation of PrP^{Sc} greatly enhanced its immunoreactivity (Serban et al., 1990) led to the development of a protocol for the detection of PrP^{Sc} (Taraboulos et al., 1990b). A speckled cytoplasmatic PrP-specific signal was then detected in scrapie infected cells that colocalized with a ligand of wheat germ agglutinin, a marker for trans-Golgi network and lysosomes (Taraboulos et al., 1990b). More precisely, immunoelectron microscopy techniques showed deposition of PrP^{Sc} in secondary lysosomes (McKinley et al., 1991). Pulse chase experiments determined that PrP^C was the precursor of PrP^{Sc} and that PrP^{Sc} acquired protease resistance as a result of a slow post-translational process (Borchelt et al., 1990; Caughey and Raymond, 1991). Acquisition of this abnormal property was not impaired by the inhibition of glycosylation (Taraboulos et al., 1990a) and occurred after exposure of PrP on the cell

surface (Borchelt et al., 1992). In particular, PrP^{Sc} was inhibited by treatment of cells at 18°C, a temperature that impairs the endosomal pathway (Borchelt et al., 1992). In order to gain insights into the exact sites involved in the genesis of PrP^{Sc}, the effect of mutant forms of the Rab family was examined (Beranger et al., 2002). The traffic of proteins between organelles occurs through vesicular intermediates that selectively convey proteins from one compartment to another. In this contest the Rab family plays a role in ensuring accurate targeting or docking of transport vesicles with their acceptor membranes (Gonzalez and Scheller, 1999). When a dominant-negative of Rab4, which is normally involved in protein recycling from endosomes to the plasma membrane (McCaffrey et al., 2001), or a constitutively activated Rab6a, which normally stimulates retrograde transport from Golgi to the ER (Martinez et al., 1994) were overexpressed in scrapie-infected N2a cells, a marked conversion of PrP^C to the pathogenic form PrP^{Sc} was detected (Beranger et al., 2002). These results showed that retrotransport of PrP^C toward the ER increased the production of PrP^{Sc} and suggest that this organelle plays an important role in the conversion process of PrP to PrP^{Sc}.

1.5.2.2 Transfected cells

Models of familial diseases have been developed by constructing stably transfected cell lines that express PrP molecules carrying mutations associated with human genetic forms, such as the point mutations P102L, D178N, E200K, Q217R or the insertional mutation of 9 octapeptide (Chiesa and Harris, 2000; Daude et al., 1997; Lehmann and Harris, 1996a; Singh et al., 1997). These forms of PrP displayed biochemical properties reminiscent of PrP^{Sc}, such as detergent-insolubility (manifested by sedimentation at 186,000 x g from

Triton/deoxycholate lysates) and protease resistance, (production of an N-terminally truncated core of 27 to 30 kDa after treatment with low doses of PK). Moreover, as part of the conversion to PrP^{Sc} state, mutant proteins became physically inaccessible to PIPLC (phosphatidylinositol phospholipase-C) release. However, these mutant molecules were distinguishable from PrP^{Sc} extracted from the brains of prion disease patients by their relatively low protease resistance and the lack of infectivity. In fact, while PrP^{Sc} purified by infected brains is resistant to high concentration of PK, mutant PrP expressed by cells is resistant only to mild concentration of the enzymes (50-100 µg/ml versus 0.5-2 µg/ml) and is not able to produce disease when injected into laboratory animals (Dr David Harris, personal communication). The mild resistance to PK would account for the difficulty in recovering the 27-30 kDa fragment after digestion of mutant PrPs, thus justifying the lack of PK resistance found in some cell models (Petersen et al., 1996).

In transfected cells, as in scrapie-infected models, conversion of PrP^C to PrP^{Sc} has been found to be a post-translational event (Borchelt et al., 1990; Daude et al., 1997). Intermediate biochemical steps in the conversion process have been identified by measuring the kinetics with which three PrP^{Sc}-related properties (PIPLC resistance, detergent insolubility and protease resistance) develop in pulse chase labelling experiments. It was found that mutant PrP becomes resistant to PIPLC treatment early after synthesis, in the endoplasmic reticulum, probably reflecting a conformational change. The second step in the conversion process is acquisition of detergent insolubility followed by acquisition of protease resistance. These last two steps develop in a post-Golgi compartment, either upon arrival at the plasma membrane or along an endocytic pathway (Daude et al., 1997).

The localisation of mutant PrP was studied by light and electron microscopic techniques. Despite equivalent expression levels by Western blotting, all cells expressing mutant PrP showed much weaker surface staining than those expressing wild-type PrP (Capellari et al., 2000; Gu et al., 2003b; Ivanova et al., 2001; Jin et al., 2000; Negro et al., 2001; Zanusso et al., 1999). To visualize intracellular PrP, fixed cells were permeabilized with Triton-X 100 prior to application of primary and secondary antibodies. In cells expressing wild-type PrP, staining was restricted to the perinuclear Golgi, a localisation that probably reflects slow transit in this compartment on the way to cell surface. In contrast, many cells expressing mutant PrP showed a much more widespread pattern of staining that colocalized with ER markers. These results suggest that several different pathogenic mutations share the property of impairing delivery of PrP molecules to the plasma membrane and cause their partial accumulation within the ER (Capellari et al., 2000; Gu et al., 2003b; Ivanova et al., 2001; Jin et al., 2000; Negro et al., 2001; Petersen et al., 1996; Zanusso et al., 1999).

1.5.3 Animal models

Animals from different species have represented essential models to study infectious and genetic prion diseases. Experiments conducted in sheep and primates in 1960's allowed demonstration of the transmissibility of scrapie, Kuru and CJD (Cuille and Chelle, 1939; Gajdusek et al., 1966; Gajdusek and Gibbs, 1971; Gajdusek et al., 1967) and to study the different route of infection (Dickinson et al., 1974; Duffy et al., 1974; Kimberlin and Walker, 1978). Moreover, infection of sheep and mice allowed investigation of the PrP genetic variability that determines the susceptibility of sheep to develop scrapie

(Dickinson and Meikle, 1971; Dickinson et al., 1968a; Dickinson et al., 1968b; Hunter et al., 1997a; Hunter et al., 1997b).

Transgenic technology has provided the means to generate lines of mice in which PrP gene is ablated or in which the mice express a modified or mutated form of the protein. These models allow studies not only of the effect of gene manipulation on mice phenotype but also allow the demonstration that PrP expression is necessary to develop disease after prion infection and the delineation of the regions of PrP that are dispensable for susceptibility to infection.

Finally, models of infectious and genetic prion diseases constitute an useful tool for testing therapeutic approaches *in vivo*.

1.5.3.1 Conventional mice and other animals

In animal models of prion diseases, the most rapid and most efficient method for inducing spongiform encephalopathy in the laboratory is the intracerebral inoculation of brain homogenate. In addition, prion diseases can be transmitted by feeding (Anderson et al., 1996), by intraperitoneal injection (Kimberlin and Walker, 1978) as well as by intraocular infection (Scott et al., 1993a) and corneal grafts (Duffy et al., 1974). Over many years, the results of oral challenge have suggested that immune cells might be of importance in the peripheral pathogenesis of prion disease. An early rise in prion infectivity can be observed in the distal ileum of infected animals (Wells et al., 1994), where Peyer's patches acquire strong immunopositivity for the prion protein. Immunohistochemical stains with antibodies to the prion protein typically reveal a robust signal in primary B-cell follicles and germinal centres, in a wide variety of secondary lymphoid organs including appendix and tonsils (Hill et al., 1997b).

All the above evidence suggest that Peyer's patches may represent a portal of entry for orally administered prions, at least in scrapie, CWD and vCJD. Normal prion protein is expressed at moderate levels in circulating lymphocytes (Cashman et al., 1990). Innate or acquired deficiency of lymphocytes impaired peripheral prion pathogenesis, whereas no aspects of pathogenesis are affected by the presence or absence of lymphocytes upon direct transmission of prion to the CNS (Lasmézas et al., 1996a). In particular, the presence of B-cells (but not expression of prion protein by these cells) is indispensable for pathogenesis (Aguzzi, 1997; Klein et al., 1997).

An interesting discrepancy that remains to be addressed concerns the actual nature of the cells that replicate and accumulate prions in lymphoid organs. Lymphocytes may be important for trafficking prions within lymphoid organs, but they do not appear to represent the major hideout for the infectious agent. Follicular dendritic cells (FDC) are a prime candidate prion reservoir, since they express large amounts of PrP and PrP accumulation tends to co-localize with FDCs in light and electron microscopical analysis (Jeffrey et al., 2000b). Moreover, it has been shown that FDC depletion impairs PrP^{Sc} accumulation in the spleen after peripheral inoculation (Montrasio et al., 2000).

Experiments performed since the early 1960s have demonstrated that transmission of prion disease from one species to another is considerably less efficient than within species, and hence the term "species barrier" was coined by Pattison in 1965 (Pattison, 1965). The effect of the species barrier is to lengthen the mean incubation period and to reduce the fraction of inoculated animals succumbing to clinical disease. To explain these phenomena it was argued that the incubation period could be so long as to exceed the natural life-span (Dickinson et al., 1975). When, following transmission across a species

barrier, brain homogenates from the recipient is passaged through the same species, the incubation period shortens and become much more consistent (Hill et al., 2000; Kimberlin and Walker, 1978; Zlotnik, 1965). First attempts to cross the species barrier were conducted with rats inoculated with sheep scrapie or mouse scrapie (Chandler and Fisher, 1963). None of the rats infected with sheep scrapie developed any sign of disease, even if analysis of brain showed vacuolation and degeneration. Conversely, one animal infected with mouse infectious material showed symptoms and lesions suggestive of experimental scrapie. In the second passage, all rats inoculated with the infected rat brain developed the disease, demonstrating the transmissibility of mouse scrapie to rats (Chandler and Fisher, 1963). Other crucial findings came from experiments with wild-type mice inoculated intracerebrally with high doses of 263K or Sc237 hamster prions. Even if the animals did not develop clinical disease 1-2 years after inoculation, the brains contained high levels of prions that, when inoculated into further mice and hamsters, caused clinical prion disease. The PrP^{Sc} found in the brains of the inoculated mice was derived from mouse and not hamster PrP^C, suggesting replication of mouse PrP^{Sc} (Hill et al., 2000; Kimberlin and Walker, 1978). These results suggest that, because of the differences in the primary sequence between PrP^C and PrP^{Sc}, the conversion rate is very slow and the accumulation of PrP^{Sc} does not reach toxic levels during the lifespan of the animals. Supporting these data, when "heterologous" cell-free conversion reactions have been performed using PrP^{Sc} of one species and PrP^C of another, striking sequence specificity was observed, which correlated with known susceptibilities of hosts to cross-species infection (reviewed in section 1.5.1).

Although a strong species barrier has frequently been observed when prions are transferred to one mammalian species to another, BSE prions have been transmitted to a variety of other species, where, in some cases, a relatively low barrier to clinical disease was observed. Transmission experiments of BSE and vCJD to mice lead to the demonstration that vCJD is derived from BSE. In fact, incubation periods and lesion profiles of mice infected with vCJD sources were closely similar to those of mice infected with BSE (Bruce et al., 1997).

Conventional mice have been extensively used to study the role of the host in transmission of prion diseases, by inoculating animals that differed by genetic background. Early experiments with inbred mice identified the *Sinc* (acronym for scrapie incubation time) gene as important in host susceptibility to scrapie (Dickinson and Meikle, 1971; Dickinson et al., 1968a; Dickinson et al., 1968b) and subsequently *Sinc* was found to be the gene encoding PrP (Hunter et al., 1987; Moore et al., 1998). In mice, only two alleles of the PrP gene have been identified (designated a and b), encoding proteins that differ by two aminoacids at codon 108 and 189 (Westaway et al., 1987). Mice with short incubation time possess codons 108 and 189 that encode leucine and threonine. Conversely, mice with long incubation time possess codons at these positions that encode phenylalanine and valine. It has been shown that each strain of TSE agent interacted with the PrP gene in a complex way, with each strain producing a characteristic and highly reproducible pattern of incubation period in the three possible PrP mouse genotypes, i.e. the two homozygotes and the heterozygote (Bruce and Fraser, 1991; Dickinson and Meikle, 1971).

1.5.3.2 Transgenic mice

The possibility of ablating endogenous genes or introducing foreign genes into the germ line of mammals has allowed the creation of further animal models of prion disease. As described in Section 1.1.3, transgenic technology has been used extensively to study the function of the prion protein and its influence on disease propagation, through gene ablation.

Transgenetics has been also used to investigate scrapie incubation times in mice with different wild-type PrP gene copy number and therefore different PrP expression levels. This research has identified the importance of gene dosage in shortening the incubation time of prion protein diseases (Westaway et al., 1991) and led to the discovery that a number of lines of transgenic mice, bearing high copy number of a wild-type PrP transgene, develop a late onset neurological disorder characterized by spongiform changes in the CNS (Westaway et al., 1994). These observations imply that overexpression of wild-type PrP is sufficient to induce a neurological syndrome similar to prion disease. In contrast, mice ablated of the PrP gene are instead resistant to prion infection, implying the absolute requirement of PrP expression to confer susceptibility to prions (Bueler et al., 1993; Manson et al., 1994b)

Mutations in the PrP coding region have also been engineered into mice in order to mimic familial forms of human prion diseases. The first mutation studied was the proline>leucine substitution at codon 102, linked to GSS. Tg(P101L) mice, expressing high level of the mouse homologue of the P102L, developed a spontaneous neurological disease (Hsiao et al., 1990). Symptoms included ataxia, spongiform degeneration, gliosis but little PrP plaque formation in the brain. PrP^{Sc} was low or undetectable and brain extracts from

spontaneously sick mice did not transmit neurodegeneration to wild-type mice. Conversely, animals expressing a lower level of the mutant protein (Tg196 mice) did not develop spontaneous disease but were reported to develop a neurological syndrome when inoculated with Tg(P101L) brain expressing high level of PrP (Hsiao et al., 1994; Tremblay et al., 2004). These animal models were obtained by traditional transgenic methods, where the insertion is based on nuclear injection into a one-cell embryo of a DNA segment containing the ORF. Of interest, the DNA segment usually concatenates prior to integration, resulting in random insertion of multiple gene copies into one or few sites of the genome, with the result that each transgenic line is unique. Depending on the site of insertion, expression of the transgene can be silenced or modulated by neighbouring regulatory elements (Hatada et al., 1999).

An alternative approach was to create the point mutations P101L in mice through a 2-stage homologous recombination in embryonic stem (ES) cells so that only endogenous level of PrP would be made under control of PrP promoter (Manson et al., 1999). This line did not shown clinical signs of neurodegenerative disease for up to 899 days of age but showed altered incubation times to disease in response to GSS prion when compared to wild-type mice.

The difference in the expression level explains the apparent discrepancies of the results obtained in all the Tg(P101L) mice described above. It is conceivable that if Tg(P101L) mice expressing low level of the mutant PrP lived longer they might develop spontaneous disease. Such a view is supported by the fact that humans with known pathogenic mutations do not develop disease until late in life. For the same reason overexpression of mutant PrP may accelerate the process of spontaneous conversion of PrP carrying

pathogenic mutation, which will manifest as disease in the lifetime of mice. Moreover, this hypothesis explain why inoculation of Tg(P101L) mice expressing a low level of PrP with the brain from spontaneously ill Tg(P101L) caused the appearance of the disease phenotype.

The expression level of the mutant transgene modulated disease in the case of Tg(PG14) mice. These mice expressed mouse PrP molecules containing an insertion of 9 additional octapeptide repeats that is associated with a familial prion disorder in humans. Two Tg(PG14) independent lines expressing the mutant PrP at the physiological PrP level developed a spontaneous neurological illness characterized by ataxia, cerebellar granule cells loss and accumulation of an aggregated and protease-resistant form of mutant PrP (Chiesa et al., 1998). The time of onset and the course of the disease were related to the expression level: mice expressing 1 fold the physiological PrP level developed the disease after ~230, while mice expressing 2 fold the physiological level developed the disease after ~60 days. Conversely, Tg(PG14) mice expressing 0.15 fold did not develop spontaneous disease (Chiesa et al., 2000; Chiesa et al., 1998). The mutant PrP form accumulating in spontaneously ill mice was mildly PK-resistant and, although it was clearly pathogenic, it did not appear to be infectious. This and other observations (see section 1.6) led to the hypothesis that pathogenicity and infectivity are distinct properties of the prion protein (Chiesa et al., 2003).

As mentioned above (see section 1.1.2), the prion protein can be synthesized in several topological forms. The role of these different forms was explored with transgenic mice expressing mutations (related to human diseases, such as A117V or artificial mutations, like KH→II , Δ STE, AV3 and G123P) that had been shown *in vitro* to alter the relative ratios of the topological

forms (Hegde et al., 1998). Transgenic mice carrying the A117V, KH→II or AV3 mutations showed a marked increase in the Ctm PrP form and developed clinical signs of neurological disease, including ataxia and paresis. The brains of the sick mice were found to contain Ctm PrP and not PrP^{Sc}. Conversely, Tg(Δ STE) and Tg(G123P) synthesized only PrP that transited normally through the ER (Sec PrP) and did not shown any signs of illness (Hegde et al., 1998). Thus, this specific transmembrane form of PrP can confer neurodegeneration in mice with features typical of prion disease.

1.6 Pathogenicity of prion protein

According to the prion hypothesis, PrP^{Sc} is the infectious form of PrP but there is no evidence that PrP^{Sc} itself corresponds to the pathogenic form that ultimately kills neurons *in vivo*. Since depletion of PrP does not induce pathology typical for prion diseases (Bueler et al., 1992), it is likely that PrP gains some toxic function upon its conversion to PrP^{Sc}, or some other abnormal forms of PrP. The most compelling evidence that PrP^{Sc} is the primary toxic entity in prion diseases is the temporal and anatomical correlation that is often observed between accumulation of this isoform and the appearance of neuropathology and clinical symptoms (DeArmond et al., 1987; Jendroska et al., 1991; Williams et al., 1997). In fact, PrP^{Sc} as detected by immunocytochemistry or Western blotting, usually appears prior to development of neuropathological changes. As the amount of PrP^{Sc} increases during the course of illness, the pathology becomes more severe. Neurografts have been used to address the question of whether the presumed toxic effect of PrP^{Sc} is related to its generation within neurons or its accumulation in the extracellular

space (Brandner et al., 1996a; Brandner et al., 1996b). In these experiments, embryonic neurons from PrP^C expressing mice were transplanted into the brains of *Prn-p*^{0/0} mice; when mice were inoculated intracerebrally with scrapie, PrP^{Sc} was generated only in the neuronal graft. Strikingly, substantial amount of PrP^{Sc} spread from the graft site into the adjacent host tissue, but did not caused pathological changes. Recently it has been demonstrated (Mallucci et al., 2003) that depleting endogenous neuronal PrP^C in conditional PrP knockout mice with established neuroinvasive prion infection reversed early spongiform change and prevented neuronal loss and progression to clinical disease. This occurred despite the accumulation of extra neuronal PrP^{Sc} to levels seen in terminally ill wild-type mice. One interpretation of these results is that PrP^{Sc} deposited extracellularly is not deleterious to neurons. Alternatively, extracellular PrP^{Sc} is toxic only once it exceeds a threshold level, but only to cells expressing PrP.

Although accumulation of PrP^{Sc} is often temporally and spatially associated with the development of pathology, several situations have now been described where this correlation is weak, or where neurodegeneration occurs in the presence of low or undetectable levels of PrP^{Sc}. A first example in which correlation between PrP^{Sc} and occurrence of pathology is weak relates to scrapie transmission to mice expressing high level of PrP. Tga20 mice that express approximately 10 times more PrP than normal mice displayed a dramatically shortened incubation period when challenged with scrapie prions (Fischer et al., 1996). However, the rate of accumulation of PrP^{Sc} in the brains of Tga20 mice did not correlate with the shortened incubation time and course of the illness. In terminally ill animals, the amount of PrP^{Sc} detectable by Western blotting was only about 50% of the amount found in infected, wild-type mice but the prion infectivity titer was equivalent (Fischer et al., 1996). Another

example involves transmission of GSS prions to mice in which both copies of the endogenous PrP gene have been replaced with one harbouring the GSS associated P101L mutation (this Tg(P101L) model has been described in section 1.5.3.2). In this case, the mice became clinically sick and developed vacuolar degeneration, but very low levels of PrP^{Sc} were present by Western Blotting and immunocytochemistry (Manson et al., 1999). Finally, C57BL/6 mice infected with the BSE agent exhibited neurological symptoms and neuronal death, but the majority of them had no detectable level of conventional PrP^{Sc}. Despite the absence of PrP^{Sc}, the cerebral tissue of these mice could transmit the disease when inoculated in a second series of mice (Lasmezas et al., 1997).

Inherited forms of prion disease confirm that the presence of PrP^{Sc} is dispensable for the occurrence of the disease. For example, patients with GSS caused by the A117V mutation lack detectable PrP^{Sc} (Piccardo et al., 2001; Tagliavini et al., 2001). Another kind of pathologic PrP that is cleaved by PK at both the N- and the C-termini, has been found in the brains of A117V patients to yield a 7 kDa fragment, considerably smaller than the 27-30 kDa PrP derived from PrP^{Sc}. Truncated forms are seen even without PK digestion and are the major component of the amyloid plaques seen in GSS patients carrying different mutations (Petersen et al., 1996). These results indicate that at least some mutations may cause disease by altering the conformation of the PrP molecule in a way that is distinct from that induced by interaction with PrP^{Sc}. Analogous to familial prion diseases of humans, the spontaneous illness developed by several kinds of PrP transgenic mice have provided support for the possibility that neurodegeneration can occur in the absence of PrP^{Sc}. Thus Tg(P101L) mice show typical clinical features of murine scrapie but without accumulation of

PrP^{Sc} in the brain (Hsiao et al., 1990). Similarly, Tg(PG14) expressing a prion protein with a nine octapeptide insertion develop a spontaneous neurological disorder characterized by the accumulation in the brain of a weakly PK-resistant form of mutant PrP (Chiesa et al., 1998).

The evidence that neuropathology can develop in the apparent absence of PrP^{Sc}, and that PrP^{Sc} can accumulate without causing clinical symptoms, argues that PrP^{Sc} might itself not be neurotoxic, and that molecules other than PrP^{Sc} could be responsible for prion-induced neurodegeneration. In principle, such neurotoxic species could be intermediates in the prion replication process that may or may not be infectious. The existence of a PrP^C–PrP^{Sc} conversion intermediate, designed PrP^{*}, has been hypothesized based on theoretical considerations (Cohen et al., 1994). Another possibility is that pathology is triggered by an altered oligomeric form of PrP, that is distinct from PrP^{Sc}. While Tg(PG14) mice express a PrP form that is weakly protease resistant and non infectious, inoculation of these mice with prions of the RML strain induced accumulation of a form of PG14 PrP that was infectious and highly protease resistant (Chiesa et al., 2003). These PrP forms, designed respectively PG14^{spn} and PG14^{RML}, were shown to differ profoundly in their oligomeric state with PG14 aggregates being much larger and more resistant to dissociation (Chiesa et al., 2003).

Several pieces of evidence have shown that the altered conformational state of PrP and its aggregation are necessary to trigger toxicity *in vitro*. PrP^{Sc} purified from infected brain has been shown to trigger apoptosis in N2a cells (Hetz et al., 2003). However, disruption of β -sheet structure completely abolished the toxicity of the protein (Hetz et al., 2003). Another support to the connection between neurotoxicity and structure/aggregation came from the use

of synthetic peptides, homologous to different fragments of prion protein. PrP(106-126) has been reported to be toxic to hippocampal and cerebellar granule neurons, neuroblastoma and PC12 cells (Fioriti et al., 2004; Forloni et al., 1993; Hope et al., 1996). Toxicity was associated to the β -sheet content and to the aggregation state of the peptide (Hope et al., 1996; Jobling et al., 1999; Rymer and Good, 2000).

Recent studies have indicated that the transmembrane form of PrP^{Ctm}PrP may play a key pathogenic role in some prion diseases. As described in sections 1.1.2 and 1.5.3.2 this form is generated in small amounts as part of normal biosynthesis of PrP (Hegde et al., 1998). However, mutations within or near the transmembrane domain, including A117V linked to GSS as well several "artificial" mutations, increased the relative proportion of ^{Ctm}PrP (Hegde et al., 1998; Hegde et al., 1999; Stewart et al., 2001; Stewart and Harris, 2001). Several lines of *in vivo* data also suggest that ^{Ctm}PrP plays an important role in the pathogenesis of some prion diseases. First, transgenic mice carrying one of the ^{Ctm}PrP-favouring mutations developed a scrapie-like neurological disease, characterized by ataxia and paresis without accumulation of PrP^{Sc}. Neuropathological examinations of brains showed vacuolar degeneration of the grey matter neuropil and astrocytic gliosis (Hegde et al., 1998). The disease was not an effect of the high copy number of the transgene, being the highest copy number present in Tg(WT) mice that remained healthy. Second brain tissue from patients with the A117V mutation, where there is a reported lack of conventional PrP^{Sc} (Piccardo et al., 2001; Tagliavini et al., 2001), was found to contain detectable ^{Ctm}PrP (Hegde et al., 1998). Third, when transgenic mice expressing wild-type hamster PrP were inoculated with mouse prions, the amount of ^{Ctm}PrP as well as PrP^{Sc} were found to increase during the course of

illness (Hegde et al., 1999). Thus, ^{Ctm}PrP could be increased either by mutations in the PrP molecule or via formation of PrP^{Sc} , leading to neurodegeneration. Although it is clear that ^{Ctm}PrP favouring mutations cause a neurodegenerative phenotype when expressed in Tg mice (Hegde et al., 1998), the data about the accumulation of ^{Ctm}PrP in scrapie infected animals is not clear (Hegde et al., 1999). The published experiment involved the use of a PrP reporter construct to monitor ^{Ctm}PrP levels, and the maximal increase in ^{Ctm}PrP was only 3-fold, much smaller than the increase in PrP^{Sc} . Other data indicate that scrapie infection of N2a cells and mouse brain does not alter the amount of ^{Ctm}PrP , thus excluding that ^{Ctm}PrP is the proximate cause of neurodegeneration in infectious prion diseases (Stewart and Harris, 2003).

Recently, it has been shown that forced cytoplasmic expression of PrP in transgenic mice by microinjecting a mouse PrP construct ablated of the signal peptide, leads to severe and rapid onset of neurodegeneration (Ma et al., 2002). The toxicity of cytoplasmic PrP (cyPrP) appeared to be selective to neurons as other tissues expressing cyPrP (such as heart) did not show pathology. In addition, cell-type specific toxicity was recapitulated in culture, where only neuronal-derived cell lines were susceptible to cyPrP-mediated apoptosis (Ma et al., 2002).

Like other membrane proteins, PrP enters the ER concurrent with its synthesis by membrane-bound ribosomes and for this reason nascent PrP cannot normally access the cytoplasm. During or shortly after its entry into the ER, PrP undergoes folding and maturations events (see section 1.1.2). It is plausible that some proportion of nascent PrP would fail to mature properly, making it a target for ER quality-control systems (Ellgaard and Helenius, 2003). These are common pathways in which misfolded proteins are recognized and

triaged for ER-associated degradation (ERAD), a process that involves retrograde translocation of substrates into the cytoplasm (possibly through the Sec61 translocon) where they are ubiquitylated and destroyed by proteasome (Tsai et al., 2002). Evidence to support this concept for PrP has recently been provided by studies in which proteasomal degradation is inhibited (Ma and Lindquist, 2001; Ma and Lindquist, 2002; Wang et al., 2004; Yedidia et al., 2001; Zanusso et al., 1999) or PrP misfolding is increased (Cohen and Taraboulos, 2003). Under these conditions a predominantly non-glycosylated form of PrP is observed to accumulate in the cytoplasm: this abnormal form has PrP^{Sc}-like properties, i.e. detergent insolubility and PK-resistance. Most intriguing was the capability of these molecules to self-propagate after the removal of the proteasome inhibitors. In fact, while neuronal cell death could be an indirect effect of proteasome inhibitors rather than the effect of cytotoxic form of PrP, the acquisition of abnormal biochemical properties and the capability of self-propagating constitute an important evidence that PrP^C molecules can occasionally undergo conformational change to PrP^{Sc}. These abnormal forms (that are normally degraded) were able to propagate their conformation (Ma and Lindquist, 2002), thus validating the prion hypothesis.

As cyPrP has been shown to be neurotoxic, it is conceivable that the access of PrP to the cytoplasm and its retention in the cytoplasm is the neurodegenerative trigger. Two further recent findings support this idea: 1) a mutated form of PrP (D177N), the mouse homologue of the D178N mutation linked to familial prion disease in humans, was shown to access the cytoplasm to a greater degree than did the wild-type PrP (Ma and Lindquist, 2001), and 2) mutations in a central hydrophobic region of PrP, that lead to increased generation of transmembrane forms of PrP, caused neurodegeneration in

transgenic mice (Hegde et al., 1998). Thus, some heritable mutations might, via multiple mechanisms, converge to a final pathway involving access of at least one portion of PrP to the cytoplasm (Ma and Lindquist, 2001; Ma et al., 2002; Yedidia et al., 2001; Zanusso et al., 1999). If mutant PrP retrotranslocates in the cytoplasm, the predicted size of PrP that accumulates should be of the fully processed molecule, i.e. 27 kD for the unglycosylated form. It has been demonstrated that in CHO and PC12 cells a small fraction of PrP molecules, both wild-type and mutant (the point mutation D177N, and the insertional mutation PG14) were subjected to proteasomal degradation in transfected cells by a pathway that did not involve retrotranslocation from the ER lumen (Drisaldi et al., 2003). Indeed, proteasome inhibitors in transfected cells expressing wild type (WT) or mutant PrP caused the selective accumulation of a form of PrP that was approximately 2 kDa larger than the mature unglycosylated species; this larger PrP molecule probably represented PrP molecules that resided on the cytoplasmic face of the ER membrane and that had never been translocated into the ER lumen for further processing. The failure to translocate was probably due to a slight inefficiency in PrP signal, as replacing it with a more efficient signal sequence increased the translocation (Rane et al., 2004). These PrP species reacted with an antibody that was specific for PrP molecules bearing an intact signal peptide (Drisaldi et al., 2003), indicating that these PrP proteins had not been exposed to signal peptidase, which resides in the lumen of the ER (Zwizinski and Wickner, 1980). In addition, these forms lacked N-linked glycans and a GPI anchor, shown by the fact that their migration was not shifted by treatment with glycosidases or PIPLC (Drisaldi et al., 2003). These features were therefore consistent with a lack of processing by oligosaccharyl transferase and GPI transamidase, both of which reside in the ER lumen

(Geetha-Habib et al., 1988; Udenfriend and Kodukula, 1995). In addition, topology analysis using a protease protection assay demonstrated directly that the protein was located entirely on the cytoplasmic side of the ER membrane (Drisaldi et al., 2003). Finally, immunolocalization studies indicated that it accumulated in an ER pattern after proteasome inhibitor treatment (Drisaldi et al., 2003). Collectively, these results indicated that a small fraction of PrP chains failed to be translocated into the ER lumen during their synthesis, but remained closely associated with the cytoplasmic face of the ER membrane where they were rapidly degraded by the proteasome. However, because all these results were obtained in transfected cells overexpressing PrP it is not possible to exclude the possibility that this mechanism is non-physiological. Indeed, untranslocated PrP does not accumulate in cultured primary cerebellar granule cells treated with proteasome inhibitors, implying that untranslocated PrP is unlikely to be an obligate by-product of PrP biosynthesis in neurons (Drisaldi et al., 2003). In contrast, accumulation of a single PrP isoform, lacking signal peptide, in the cytosol of some neuroblastoma cell lines (N2a, BE(2)-M17 and SK-N-SH) and in primary neurons was detected after co-treatment with brefeldin A (which blocks the transport of secreted proteins beyond the endoplasmic reticulum (Misumi et al., 1986) and the proteasome inhibitor epoxomicin (Roucou et al., 2003). However, while this form was toxic in N2a cells and showed the abnormal biochemical properties described for PrP^{Sc}, neither toxicity or alteration of biochemical properties was shown in primary neurons or BE(2)-M17 and SK-N-SH cells (Roucou et al., 2003). The physiological retrotranslocation of PrP is indeed still controversial, as another group recently reported the accumulation of a 27 kD form of the protein, depleted of the signal peptide, after proteasome inhibition in several cell lines

expressing high level of endogenous PrP and in primary cortical neurons (Wang et al., 2004).

Taken together, all the results described demonstrate that a cytosolic PrP can accumulate in different cellular models in non-physiological conditions: 1) because of impaired degradation of abortively translocated, signal peptide-bearing molecules synthesized from the CMV promoter (Drisaldi et al., 2003) or 2) favouring the accumulation of protein in the ER by brefeldin A treatment (Roucou et al., 2003) or simply by inhibiting proteasome (Wang et al., 2004). In none of the described case, however, did cytosolic PrP forms show a clear and reproducible cytotoxicity, thus making it unlikely that neurodegeneration in prion disease may ensue from abnormal accumulation of toxic PrP species in the cytoplasm.

A number of inherited human diseases are attributable to defects in export of a mutant protein from the ER. In some cases, such as cystic fibrosis and hereditary hemochromatosis (Aridor and Balch, 1999), the mutant protein is retrotranslocated from the ER and degraded by the proteasome, resulting in failure of the protein to reach its normal cellular destination. In other disorders, such as hereditary emphysema (PiZ variant) and congenital hypothyroidism, the retained protein accumulates in the ER without being degraded. In these cases, the disease phenotype is due to a toxic effect of the accumulated protein, which stimulates one or more ER stress response pathways (Aridor and Balch, 1999). It was hypothesized (Drisaldi et al., 2003) that some inherited prion disorders, such as those due to PG14 and D177N mutations, are members of this second category of ER retention diseases. Accordingly, immunofluorescence studies showed that less mutant PrP is present on cell surface compared to WT. Although transit of D177N and PG14 mutant PrP molecules out of the ER is not

completely blocked, their export rate is reduced sufficiently to cause an accumulation of the protein in the ER at steady state (Ivanova et al., 2001). Thus these ER-accumulated proteins could induce an ER-stress response (also called the unfolded protein response, UPR), which included a signalling component known to trigger molecules, such as CHOP-GADD153, promoting cell-death (Kaufman, 1999). Supporting this hypothesis, up regulation of ER stress genes such as genes encoding for glucose-regulated protein GRP58, GRP78 and GRP94 has been demonstrated in N2a cells challenged with purified PrP^{Sc}, in the brain of infected animals and of sporadic and variant CJD (Hetz et al., 2003).

1.7 The unfolded proteins response (UPR)

All eucaryotic cells have an extensive membranous labyrinth network of branching tubules and flattened sacs called the endoplasmic reticulum (ER). A large fraction of all cellular proteins are translocated into the ER where folding, oligomerisation and other post-translational modifications occur prior to transport to the extracellular surface or to cellular organelles, such as the Golgi compartment or lysosomes. Many proteins that acts as molecular chaperones or folding catalysts reside in the ER and form a matrix on which newly synthesised proteins attain their final conformation (Ellgaard and Helenius, 2003). This interaction ensures that only properly folded and assembled proteins exit the ER compartment, a process known as "quality control" (Hurtley et al., 1989). Molecular chaperones were originally defined as families of proteins that assist in the self-assembly of other chains (Ellis et al., 1989). Several chaperones are known to be members of the family of heat shock

proteins (HSPs). Heat shock is only one of several different types of stress that can cause protein denaturation and the HSP60, HSP70 and HSP90 classes of proteins, so named for their approximate molecular weight, are known to play crucial compensatory roles that allow cell survival (Craig et al., 1994).

To maintain the efficiency of quality-control mechanisms in diverse conditions, living cells have evolved regulatory circuits that monitor the levels of available chaperons. This is true for both the cytosol and the ER, and compartment-specific responses clearly exist that selectively restore optimal levels of the desired folding factors. Accumulation of aberrant proteins in the cytosol triggers the heat-shock response, resulting in the *novo* synthesis of HSP70 and other cytosolic chaperons (Morimoto, 1998). But if aberrant proteins accumulate in the ER, cells activate a different response. The ER is sensitive to alterations in homeostasis: upon a variety of different stimuli, signals are transduced from the ER to the cytoplasm and the nucleus. The immediate response occurs at the translational apparatus, whereas changes in gene expression promote long-term adaptation or apoptotic cell death (Kaufman, 1999). These stimuli include calcium depletion from the ER lumen, inhibition of asparagine (N)-linked glycosylation or of disulphide bond formation, expression of mutant proteins or protein subunits and also overexpression of wild-type proteins. When protein misfolding occurs and unfolded proteins accumulate and aggregate in the ER, the cell activates adaptive signalling pathways that are programmed to enhance the folding capabilities in this compartment. This signal response was termed the unfolded protein response (UPR) (Kaufman, 1999). The UPR is essential for survival of all eukaryotic cells under conditions of stress and is also implicated in the pathogenesis of a number of disease such as hereditary enphysema and cystic fibrosis (Aridor and Balch, 1999). The UPR

culminates in the induction of the ER stress-response genes, including those that encode several glucose-regulated proteins (GRP), such as GRP78 and GRP94, calreticulin, growth arrest and DNA damage gene 153 (GADD153), protein disulfide isomerase (PDI), and x-box-binding-protein (XBP1) (Kaufman, 1999; Lee et al., 2002). GRP78 was identified as an ER-localized protein that bound to the heavy-chain immunoglobulins and inhibited their secretion in the absence of the light chain in pre-B lymphocytes (Haas and Wabl, 1983). For this reason it was named immunoglobulin binding protein (BiP). Independently BiP was identified as a member of a protein family that is expressed at high levels upon exposure to glucose (for this reason called glucose-regulated protein, GRPs) and was named GRP78 (Shiu et al., 1977). More recently, it has been shown that BiP is identical to GRP78 (Munro and Pelham, 1986). BiP is a member of the HSP70 family and displays weak ATPase activity that is stimulated by peptides that contain hydrophobic amino acids. It interacts transiently with exposed hydrophobic patches on protein folding intermediates and it is thought to prevent their aggregation (Kassenbrock et al., 1988; Kim and Arvan, 1998). GRP94, also known as endoplasmin, is a member of the HSP90 family and is also a calcium binding protein (Kim and Arvan, 1998). This protein associates with nascent polypeptides that have exposed unfolded patches and has a weak ATPase activity. Like BiP, it is found to interact with misfolded proteins for prolonged periods after synthesis (Kim and Arvan, 1998). Calreticulin is a lectin (a protein which binds carbohydrates) and is homologous to another ER resident protein named calnexin. They both interact with monoglycosylated, trimmed intermediates of the N-linked core glycans on newly synthesised glycoproteins, thereby preventing their transit through the secretory pathway. Calreticulin is also the major calcium-binding and storage protein in

the ER (Kim and Arvan, 1998). GADD153, also known as CHOP 10 (C/EBP homologous protein 10) was originally identified as a gene induced on DNA damage and growth arrest (Kim and Arvan, 1998). However, subsequent studies have demonstrated a strong correlation between development of ER stress and induction of CHOP (Wang et al., 1996). CHOP is a small nuclear protein that can heterodimerize with C/EBP family members and activate downstream target genes which negatively regulate cell growth or induce apoptosis (Barone et al., 1994; Ron and Habener, 1992). CHOP 10 is regulated not only at transcriptional level but also at a post-translational level: on phosphorylation of serine residues by p38 MAP kinase, its transcriptional activity increases (Wang and Ron, 1996). Protein disulfide isomerase (PDI) catalyses oxidative folding and increases the rate at which proteins attain their final folded conformation (Lambert and Freedman, 1985). XBP1 is a basic leucine-zipper (b-zip) transcription factor originally identified as a protein binding to the cis-acting x-box present in the promoter regions of human major histocompatibility complex class II genes (Liou et al., 1990). XBP1 mRNA is spliced in response to ER stress to produce a highly active transcription factor that enhances transcription of genes implicated in glycoprotein degradation (Yoshida et al., 2003).

The activation of UPR has been extensively studied in yeast cells. In *Saccharomyces cerevisiae*, the proximal sensor of the UPR is the protein kinase and endoribonuclease Ire1p, the translation product of inositol-requiring 1 gene (IRE1). Ire1p is a transmembrane type I protein, the N-terminal half of which is located in the ER lumen; the protein kinase and endoribonuclease are located in the cytoplasmic side. Protein accumulation in the ER promotes Ire1p dimerization, autophosphorylation and activation of its ribonuclease activity

(RNase) (Shamu and Walter, 1996). The only substrate known is HAC1 mRNA (homologous to ATF/CREB 1') encoding the b-ZIP transcriptional factor Hac1p that binds to the UPR target genes. Ire1p cleaves HAC1 mRNA at two sites to remove a 252 nucleotide intron (Sidrauski and Walter, 1997). Hacp1 is synthesized only after Ire1p-mediated splicing occurs because the HAC1 intron has a strong ability to block translation.

Two related homologues of yeast IRE1p, referred to as IRE α and IRE1 β , have been identified in both the human and murine genome (Tirasophon et al., 1998; Wang et al., 1998). Both are type I transmembrane proteins in the ER, with their cytoplasmic regions carrying protein kinase and endoribonuclease domains. Purified IRE1 α and IRE1 β can cleave HAC1 precursor mRNA at the same sites as yeast Ire1p (Tirasophon et al., 1998). In mammalian cells, XBP1 has been identified as a substrate for endoribonuclease activity of IRE1 α and IRE1 β (Yoshida et al., 2001) (Figure 1.9).

In higher eukaryotic cells, the other pathway for gene activation upon accumulation of unfolded protein in the ER involves the activation of the activating transcription factor 6 (ATF6) (Haze et al., 1999). ATF6 is constitutively expressed in the ER as a 90 kDa type II transmembrane protein. The N-terminal half, facing the cytoplasm, contains a b-zip motif. Upon activation of the UPR, ATF6 is proteolyzed within the transmembrane domains by site-1 and site-2 (SP1 and SP2) protease to release a 50 kDa cytosolic fragment (p50ATF6). p50ATF6 migrates to the nucleus and activates transcription of ER chaperones and enzymes mainly implicated in protein quality control and folding (Figure 1.9). BiP and XBP1 are genes that are induced by overexpression of ATF6 (Yoshida et al., 1998).

Therefore, IRE1 and the ATF6 signaling pathways merge through regulation of XBP1 activity to induce downstream gene expression. Whereas ATF6 increases XBP1 mRNA, IRE1 removes the 26-nucleotide intron that increases XBP1 transactivation potential (Lee et al., 2002).

The third ER sensor is the protein kinase Perk (Liu et al., 2000; Shi et al., 1998). After trans-autophosphorylation Perk phosphorylates its only known substrate, the α subunit of eukaryotic translation initiation factor 2 (eIF2 α), to limit polypeptide chain initiation. This phenomenon is called translation attenuation and protects cells under conditions where proteins cannot fold properly. This pathway also activates ATF4, causing increased expression of genes involved in aminoacid import and redox regulation, and the CHOP/GADD153 involved in apoptosis (Harding et al., 2003) (Figure 1.9).

UPR not only plays an important role in protecting cells from adverse physiological conditions, but recent studies suggest that activation of at least a part of the UPR is involved in normal physiological processes like differentiation of B cells into plasma cells (Gass et al., 2002) and the secretion of insulin by β islet cells of the pancreas in response to changes in blood glucose levels (Harding et al., 2001).

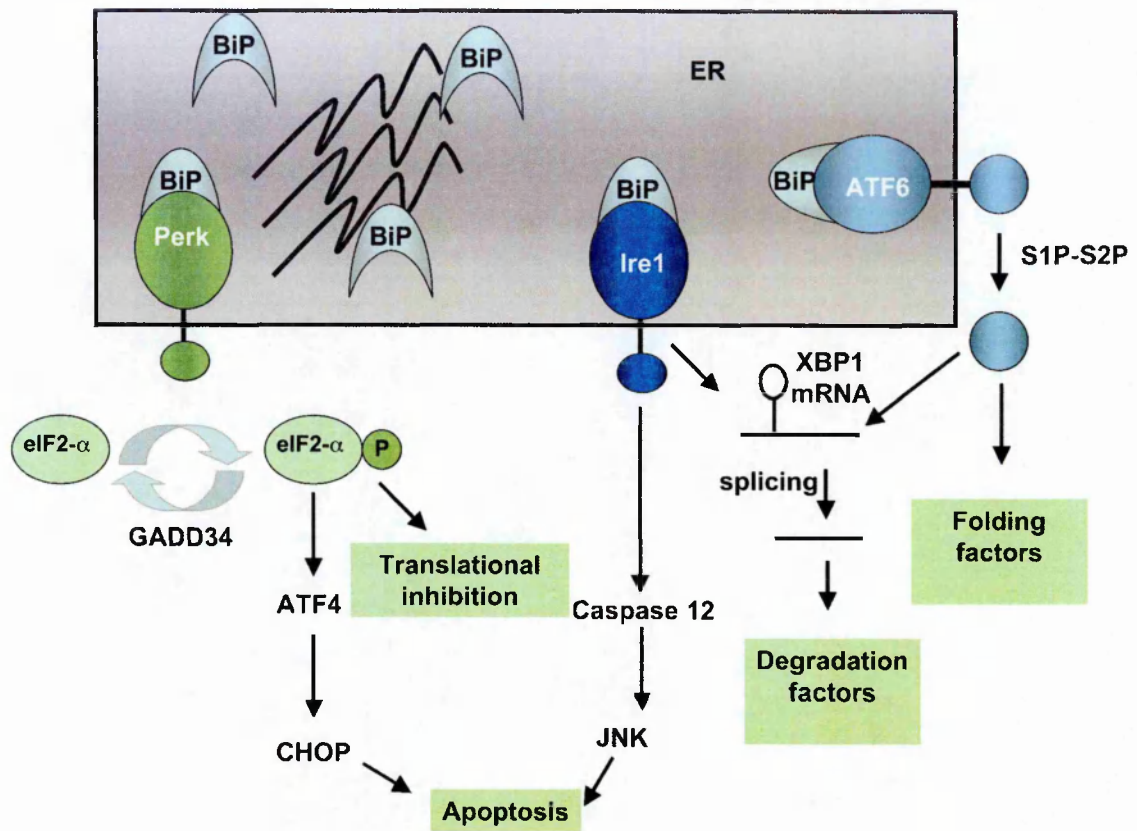


Figure 1.9: Schematic representation of UPR.

When misfolded proteins accumulate in the ER, three ER localised membrane proteins are activated, Perk, Ire1 and ATF6. Activation seems to be mediated by the release of BiP from the ER transmembrane proteins. Upon activation, Perk phosphorylates eIF2 α , causing translational inhibition. Subsequently, ATF4 is up-regulated increasing the transcription of genes involved in aminoacid import, redox regulation and apoptosis (like CHOP). Upon accumulation of unfolded proteins ATF6 leaves the ER to enter the Golgi apparatus where it is cleaved to release a 50 kDa fragment (by S1P and S2P proteases) that enter the nucleus. P50-ATF6 increases the transcription of ER chaperons. Simultaneously UPR induces activation of ribonuclease activity of IRE1. Translation of spliced XBP1 generates a potent transcriptional activator that increases transcription of genes implicated in glycoprotein degradation.

1.8 Development of inducible cellular models of familial prion diseases

As mentioned in section 1.5.3.2, neurotoxicity of mutant PrP has been demonstrated *in vivo* by generating transgenic mice lines that express altered forms of PrP linked to familial prion diseases (Chiesa et al., 1998; Hsiao et al., 1990). Conversely, mutant PrP expressed in transfected cells acquired scrapie-like properties but has not shown toxicity in any cellular type, including neuronal lines (Chiesa and Harris, 2000; Petersen et al., 1996; Priola and Chesebro, 1998; Singh et al., 1997; Zanusso et al., 1999). Several hypotheses can be proposed to explain these results. First, mutant protein could not be sufficiently "scrapie-like", or neuronal degeneration could require interaction with other cell types, such as microglia or astrocytes. Secondly, a toxic effect could require chronic exposure of cells over many months, much longer than can be achieved in culture. Finally, since PrP expression in the described models was constitutive, it was possible that only clones resistant to the toxicity of PrP expression had been selected after transfection. In fact, cells can evoke different type of responses to accommodate expression of a the toxic protein (Sherman and Goldberg, 2001).

To exclude this possibility, i.e. toxicity of the transfected protein during clonal selection, an inducible model can be established. In this case, transcription is activated after clonal selection by adding a specific inducer. Inducible systems are also particularly useful as they allow direct comparisons of the effect of protein expression on the same cellular background to be made, avoiding clonal variability. It is therefore possible to study the potential cytopathic effect of protein expression and the molecular mechanism leading to

cell death, manifested by differences in genes expression, differences in protein activation, etc.

A number of inducible promoters have been described in eukaryotic cells that are responsive to heavy metal ions (McNeall et al., 1989), heat shock (Bienz and Pelham, 1986) or hormones (Renkawitz et al., 1982; Rousseau, 1984). However, these systems have generally suffered from leakiness of the inactive state or from pleiotropic effects caused by the inducing agent itself. An example of the pleiotropic effect, i.e. the tendency to affect the expression of multiple gene other than the target, is represented by the use of steroid hormones as an inducer, known to regulate the expression of several genes. To avoid pleiotropic effects regulatory systems that do not rely on endogenous control elements have been developed: the ecdysone inducible system, based on the *Drosophila melanogaster* molting hormone (No et al., 1996), and the Tet-system, based on the tetracycline repressor of *E. coli* are those most commonly used.

1.9 Tet-on and Tet-off gene expression systems

1.9.1 How the Tet-system works

In *E. Coli*, the Tet repressor protein (TetR) negatively regulates the genes of the tetracycline-resistance operon on the Tn10 transposon. The TetR blocks transcription of these genes by binding to the tet operator sequences (tetO) in the absence of tetracycline (Tc). TetR and tetO provide the basis of the Tet-off and Tet-on for mammalian experimental systems (Gossen and Bujard, 1992; Gossen et al., 1995).

The first component of the Tet-system is the regulatory protein based on TetR. In the Tet-off System, this protein is a fusion product of aminoacids 1-207 of TetR and the C-terminal 127 aminoacids of the Herpes Simplex Virus protein VP16. The VP16 domain converts the TetR from a transcriptional repressor to a transcriptional activator. The hybrid protein is known as the tetracycline-controlled-transactivator (tTA). The second component is the response plasmid, which expresses the gene of interest under control of the tetracycline-response element, or TRE. The TRE consists of seven direct repeats of a 42-bp sequence containing the tetO, and is located just upstream of the minimal cytomegalovirus (CMV) promoter. Binding of the regulatory protein to the promoter allows transcription; when tetracycline is added to the medium it prevents the binding and causes a 10-fold decrease of the transcription within 8 h (Figure 1.10A, taken from the Tet-on & Tet-off gene expression system manual, Clontech).

The Tet-on system is similar to the Tet-off system, but the regulatory protein is based on a "reverse" Tet repressor, rtTA (reverse tTA), created by four amino acid changes in TetR. Addition of the effector doxycycline (Dox, an analogue of tetracycline) results in binding of rtTA to tetO, which allows gene activation up to 1000-fold (Figure 1.10B taken from the Tet-on & Tet-off gene expression system manual, Clontech).

The Tet-off and Tet-on system have several advantages over other regulated gene expression systems (Gossen and Bujard, 1992; Gossen et al., 1995):

1. There is extremely tight on/off regulation. Background or leaky expression in the absence of induction is extremely low (Harkin et al., 1999; Li et al., 1998) (Figure 1.10C).

2. TetR or rTetR act specifically on their target sequences, avoiding any pleiotropic effect (Harkin et al., 1999).
3. The response to the inducer is high and fast: induction can be detected within 30 minutes and reaches up to 10,000 fold induction levels (Yin et al., 1996) (Figure 1.10C).

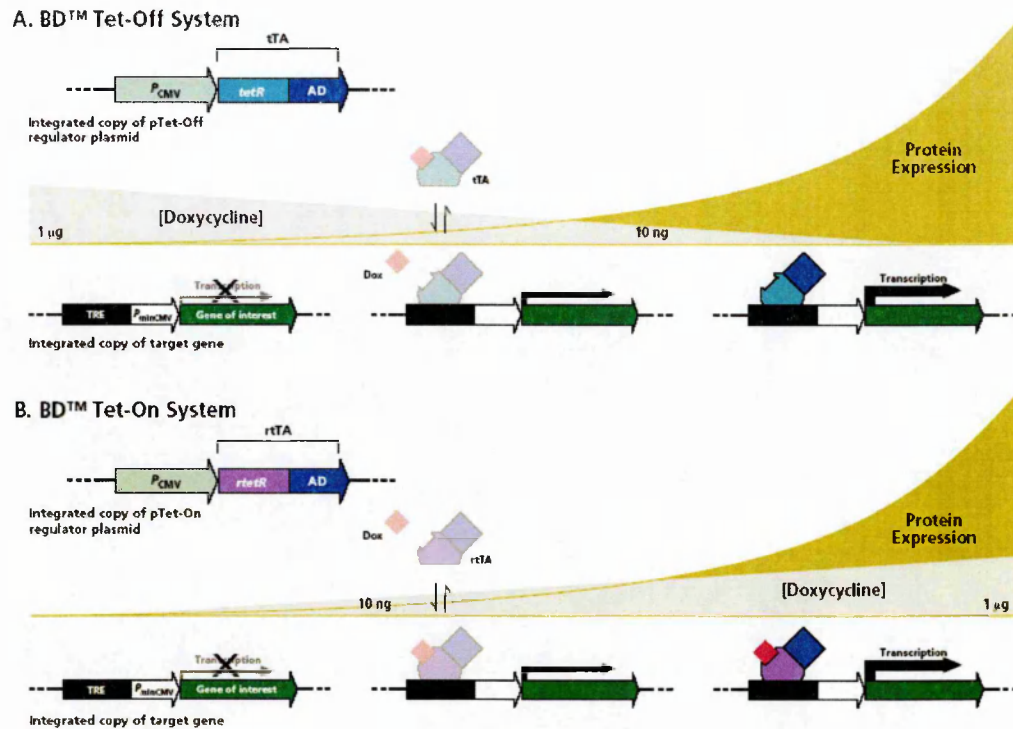


Figure 1. 10: The BD™ Tet-Off and Tet-On Systems.

(A) BD™ Tet-Off System. The Tet-controlled transactivator (tTA) is a fusion of the wild-type Tet repressor protein (tetR) to the VP16 activation domain (AD) of herpes simplex virus.

(B) BD™ Tet-On System. Four amino acid changes to TetR alter its binding characteristics and create the reverse TetR (rTetR), which binds the TRE in the presence of doxycycline.

To ensure the absence of unregulated gene expression in the Tet-on system, it is possible to introduce another element, the pTet-tTS, which encodes a transcriptional silencer (tTS) that blocks transcription of genes under control of the TRE in the absence of Dox (Figure 1.11) (Freundlieb et al., 1999). The addition of tTS reduces background expression in mammalian cell lines (Forster et al., 1999; Freundlieb et al., 1999; Rossi et al., 1998) and in transgenic animals (Perez et al., 2002; Zhu et al., 2001) even if it sometimes affects the maximal expression level (Knott et al., 2002)

Different plasmids harboring the TRE element are available: in pBI-G the promoter is bi-directional and the inducer regulates expression of the gene of interest along with β -galactosidase (Figure 1.12A). Because this plasmid does not encode for any selective agent resistance the plasmid pTK-Hyg must be co-transfected (Figure 1.12B).

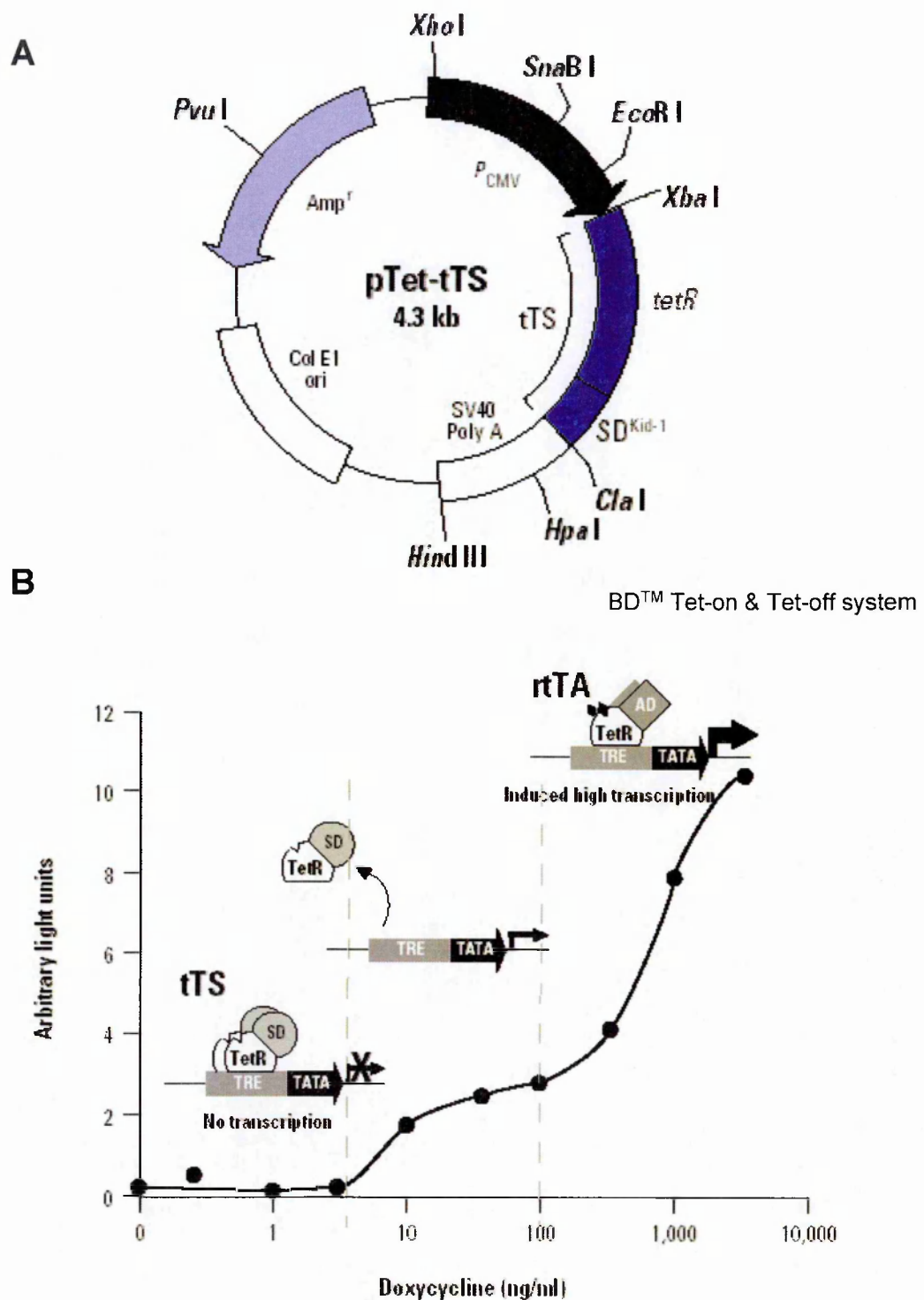
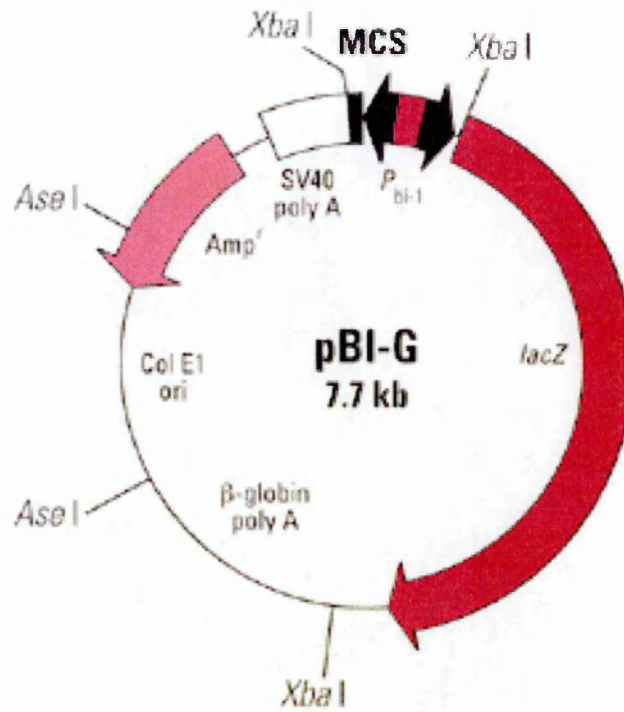


Figure 1.11: The role of pTet-tTS plasmid.

(A) Map of pTet-tTS, which expresses tTS inhibitor.

(B) Dose response curve demonstrating controlled expression in a cell line co-expressing tTS and rtTA. Cells, constitutively expressing rtTA, were transiently transfected with a plasmid expressing tTS and a control vector expressing luciferase downstream of the TRE. Cells were cultured in the indicated levels of Dox. After 24 hr, cells were harvested and assayed for luciferase activity. SD = silencing domain. AD = activation domain. Data provided courtesy of S. Freundlieb, *et al.*, Zentrum für Molekulare Biologie (ZMBH), Heidelberg.

A



B

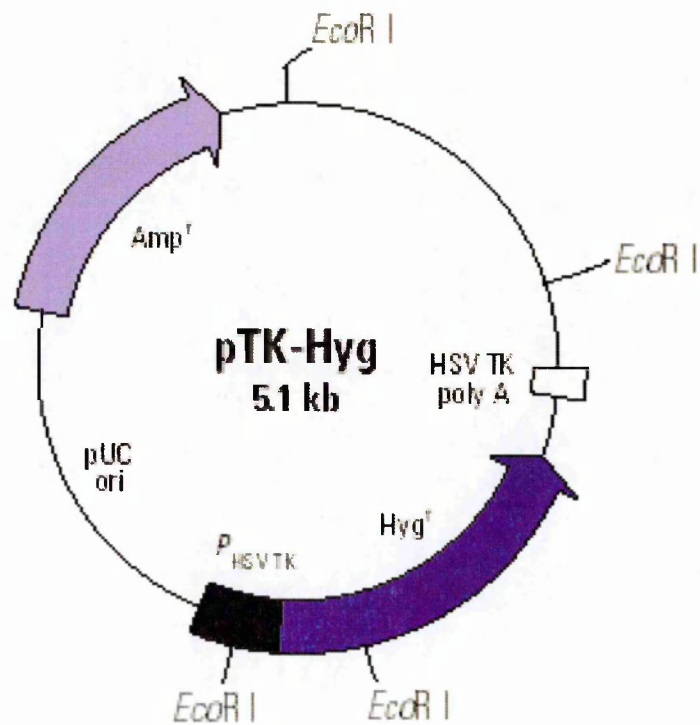


Figure 1.12: Schematic representation of pBI-G and pTK-Hyg plasmids.
 (A) Map of pBI-G, harboring the TRE element.
 (B) Map of pTK-Hyg, which confers resistance to hygromycin.

1.9.2 Applications of the tet-system

Tet inducible systems have been successfully used in cellular and animal models of other neurodegenerative diseases, such as Parkinson's disease (PD) and Huntington's disease (HD).

PD is a progressive neurodegenerative disorders caused by the loss of dopaminergic neurons in the substantia nigra (Nussbaum and Polymeropoulos, 1997). Although mutations in α -synuclein have been identified in autosomal dominant PD, the mechanism by which neuronal cell death occurs remains unknown. To study the involvement of α -synuclein in dopaminergic neuronal cell death, PC12 cell lines have been established in which wild-type or mutant protein can be derepressed by removing doxycycline (Lee et al., 2003; Tanaka et al., 2001). These studies showed that expression of mutant α -synuclein upon removal of doxycycline decreased proteasome activity and increased the sensitivity of cells to mitochondria-dependent apoptosis.

HD is a neurodegenerative disorder caused by the abnormal expansion of a polyglutamine tract in the huntingtin protein (Bates, 2003). In order to study the cellular response to mutant huntingtin, 293 Tet-off and PC12 Tet-off cells expressing wild-type or a mutated form of the protein have been established. Huntingtin aggregation led to distinct perinuclear inclusion bodies and resulted in redistribution of several cellular factors, such as stress proteins and components of the proteasome system to the inclusion bodies. Inclusion body formation also resulted in cell toxicity and ultrastructural changes such disruption of the nuclear envelope and cytoplasmic vacuolation (Igarashi et al., 2003; Waelter et al., 2001). A conditional mouse model of HD has also been generated using the tet-responsive system. Mice expressing a mutated

huntingtin developed a progressive motor dysfunction, that was associated with severe neuronal inclusions. Blockage of expression in symptomatic mice by Dox administration led to disappearance of inclusions and to amelioration of the phenotype. The use of the conditional model therefore demonstrated that the continuous influx of the mutant protein is required for pathology in HD (Yamamoto et al., 2000).

1.9.3 Use of the Tet system in cellular and animal models for the investigation of prion diseases

The Tet-on and the Tet-off systems for expression of PrP have been studied in N2a cells (Windl et al., 1999). However, the Tet-off system showed leakage with maximal PrP expression achieved irrespective of whether Dox was present in the medium. In contrast, quantitative analysis of the N2a-rtTA cells revealed an expression level that was four times higher in the induced state than in the uninduced condition and slightly higher in comparison to the expression level of PrP under control of a constitutive CMV promoter. In the absence of inducer, the expression level was indistinguishable from the endogenous PrP expression of N2a cells. Expression was directly related to Dox concentration and reversed within 96 h of Dox depletion (Windl et al., 1999). These N2a-rtTA cells were also used to demonstrate that murine PrP in which all the alanine residues of the sequence AGAAAAGA (spanning aminoacids 109-112) were replaced by glycine residues acquired PK resistance (Wegner et al., 2002).

PrP was also expressed in RK13 Tet-on cells that do not express PrP (Rachidi et al., 2003). In this model, a link between PrP^C, copper binding and resistance to oxidative stress was reported (Rachidi et al., 2003). Expression of

PrP in fact, was related to an increased concentration of copper in cell membrane and determined an increased survival after oxidative stress.

A Tet-off system was also used to control expression level of wild-type PrP in transgenic mice (Tremblay et al., 1998). Doxycycline administrated to adult Tg mice acutely repressed the expression of PrP but did not produce any recognizable adverse effects in mice. This result indicated that high levels of PrP were not essential for short-term neuronal survival and its expression could be repressed over 20-fold without effects. Upon PrP repression, mice challenged with RML prions remained free of any signs of CNS dysfunction for more than 380 days. Histological analysis showed a low level of PrP^{Sc}, probably due to the leakage of the promoter. In contrast, mice that had not been treated with Dox developed a progressive ataxia starting 50 days after inoculation with prions. These experiments provided supporting evidence that low levels of PrP^{Sc} do not produce clinical or neuropathological effects (Tremblay et al., 1998).

1.10 Work of this thesis

As discussed in Sections 1.6 and 1.8, many investigations have sought to identify the molecular basis of mutant PrP toxicity. However, none of the *in vitro* models of familial prion diseases developed to date demonstrated a clear-cut cytopathic effect of mutant PrP expression. Moreover, the pathogenic molecular mechanisms by which mutant PrP causes neuronal cell and dysfunction remain uncertain.

In an attempt to reproduce neuronal degeneration *in vitro* and to gain insights into the molecular mechanisms leading to cell death, an inducible

model of familial prion diseases was developed by expressing the mouse homologue of the D178N mutation in two forms (D177N (Met128) and D177N (Val 128) in PC12 Tet-on cells. The same insertional mutation, PG14, which has been previously characterised in PC12 cells (Chiesa and Harris, 2000) and Tg mice (Chiesa et al., 1998) was also expressed. Further, because an involvement of the UPR in prion diseases has recently been reported (Hetz et al., 2003), this model was used to evaluate differences in the expression level of UPR gene in the induce state *versus* the un-induced state. The PC12 cell line was chosen to directly evaluate the potential cytopathic effect of mutant PrP expression in cells that have acquired a neuronal phenotype, by activating transcription after differentiation with nerve growth factor (NGF). A Tet-on system was preferred, rather than a Tet-off, because of the absence of leakage of expression when combined with the tTS repressor (Forster et al., 1999; Freundlieb et al., 1999; Rossi et al., 1998).

Chapter 2: Materials and Methods

2.1 Cloning of wild-type and mutant PrP cDNAs into the Bidirectional Tet vector

Construction of cDNAs encoding wild-type (M128, WT) or PG14 PrP carrying an epitope tag for the monoclonal antibody 3F4 was previously described (Lehmann and Harris, 1995). cDNAs encoding 3F4-tagged D177N/Met128, D177N/Val128 and M128V cloned in pCDNA3 were kindly donated by David Harris, Washington University, St. Louis, Missouri, USA. Pst I and Not I restriction sites were inserted respectively upstream the 5' and downstream the 3' ends of the PrP open reading frame by polymerase chain reaction. The products of amplification were cloned into the Pst I/ Not I restriction sites of the Bidirectional Tet Vector pBI-G (BD Clontech, Palo Alto, California), containing the Tetracycline-Response-Element (TRE) (Figure 2.1).

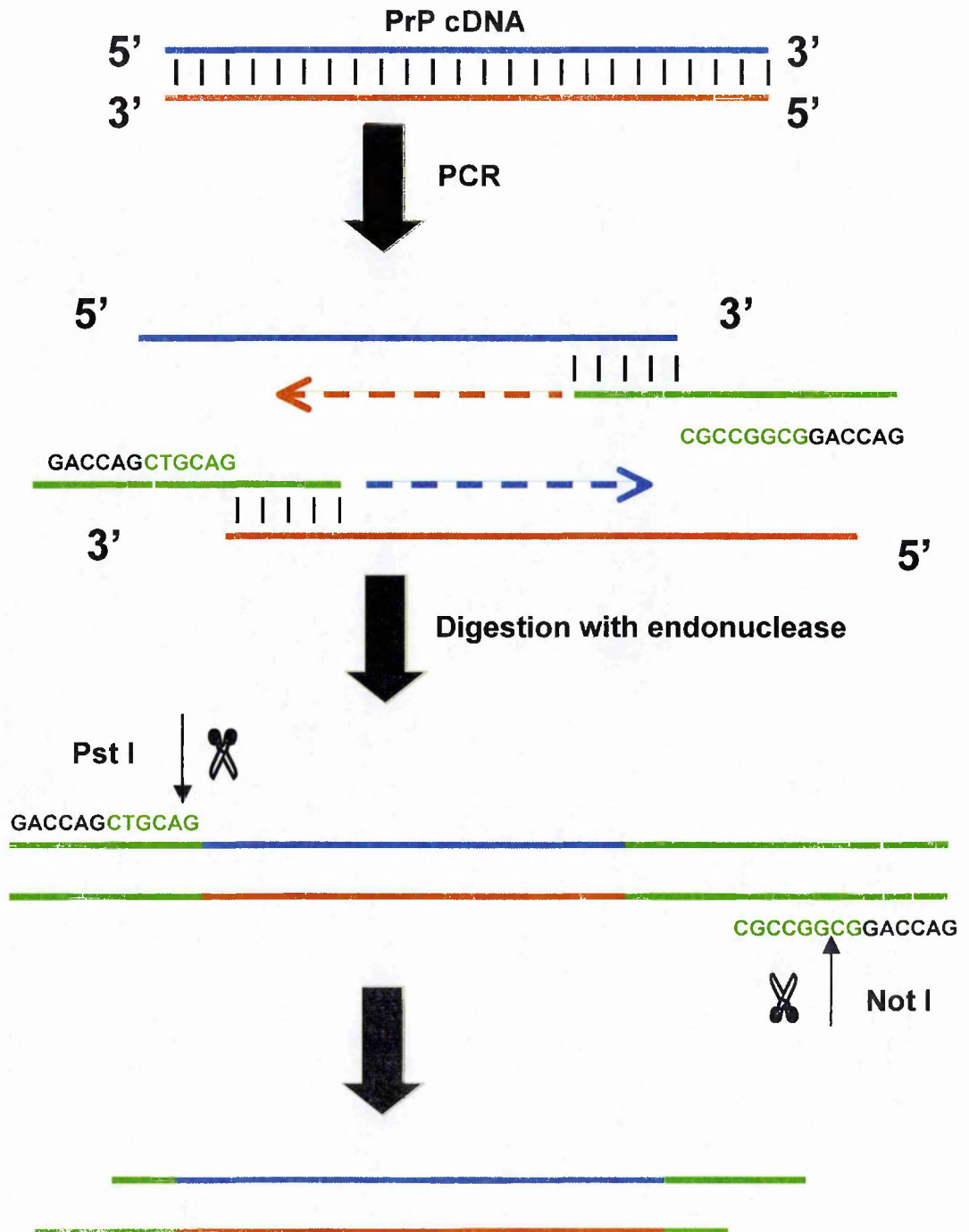
For the PCR, two oligonucleotides were synthesized. The following criteria were followed to design the primers: 1) the length of the region annealing to either the 5' or 3' end of the PrP cDNA were chosen to have a melting temperature (T_m) of approximately 60°C ; 2) the consensus sequences for the restriction enzymes were inserted at the 5' end of each primer and were followed by a GC-rich sequence which favours the annealing of the primers after the first PCR cycle, and constitute a spacer at the 5' end of the restriction site to facilitate binding of the restriction enzyme; 3) T_m of the full length primer of approximately 65°C. The sense primer was: 5'-

GACCAGCTGCAGATGGCGAACCTTGGCTACTGGCTG-3'; the antisense primer was: 5'-GACCAGGCGGCCGCTCATCCCACGATCAGGAAGATGAG-3'. Starting from 5', each primers is composed by the CG rich sequence GACCAG, by the restriction site Pst I or Not I and by the PrP sequence (aminoacids 1-8 or 248-254 plus stop cordon). The PCR was performed in a thermal cycler (PTC-200; Mj RESEARCH, Inc., Waltham, Massachusetts) for 20 cycles at 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s. 10 ng of template DNA was added to 50 µl of reaction mixture containing 10 mM KCl, 20 mM Tris-HCl pH 8.8, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, 0.2mM dNTPs (Fisher Scientific UK Ltd, Leicestershire, Loughborough, England), 0.5 µM primers and 2 U of Vent polymerase (New England BioLabs, Inc., Beverly, Massachusetts), which possess 3' exonuclease proof-reading activity. The calculated length of the amplified fragments was 791-bp band for the WT, M128V, D177N and 1008 bp for the PG14 construct (12 nucleotides of the sense primer + 762 or 979, which is the length of the entire cDNA sequence (762 for the WT, M128V and D177N PrP, and 979 for PG14 PrP) + 17 nucleotides of the antisense primer) (Figure 2.1 A).

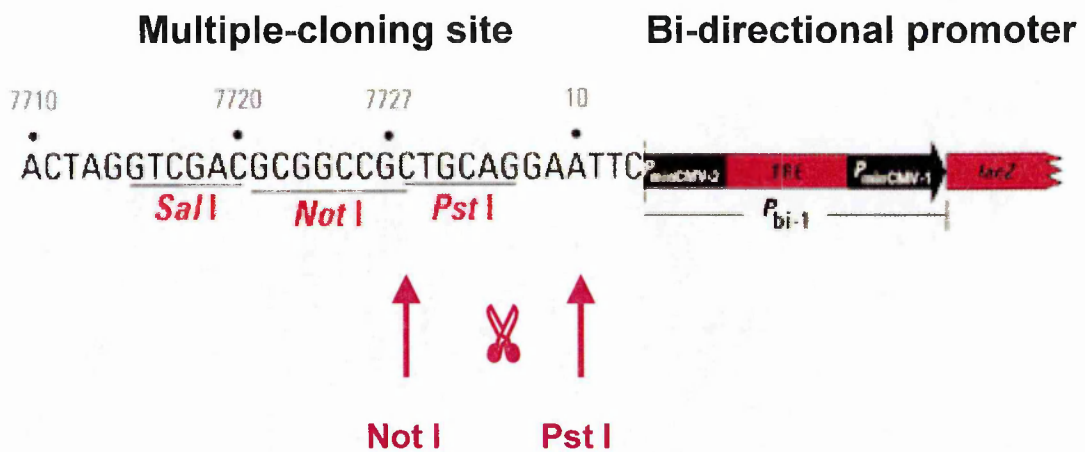
The PCR products were digested with 2 U/µg of Not I (Roche Diagnostics, Mannheim, Germany) for 2 h at 37°C and subsequently digested with 2 U/µg of Pst I (Roche Diagnostics, Mannheim, Germany) for 2 h at 37°C (Figure 2.1A). Digested samples were separated onto a 0.5% low-melting agarose gel and purified by ELUTIP minicolumns (Schleicher & Schuell Bioscience Inc., Keene, Nevada). 10 µg of pBI-G were digested with 20 U of Not I and Pst I for 2 h at 37°C and dephosphorilated by further 1 h incubation with 2 U of alkaline phosphatase (Roche Diagnostics, Mannheim, Germany) (Figure 2.1B). Linearized-dephosphorilated vector was isolated from low-melting

agarose gel with ELUTIPS minicolumns. For the reaction of ligation, 500 ng of insert DNA and 100 ng of linearized, desphosphorilated pBI-G were incubated with 1 U of bacteriophage T4 DNA ligase (Roche Diagnostics, Mannheim, Germany) for 18 h at 16°C (Figure 2.1C). As a negative control 100 ng of linearized pBI-G were incubated with 1 U of ligase in the absence of insert DNA.

A



B



C



Figure 2.1: Strategy for PrP cDNA cloning.

(A) PrP cDNAs containing Pst I and Not I restriction sites were generated by PCR. The PCR product was subsequently digested with Pst I and Not I. PrP cDNA is shown in red/blue. Primers are shown in green. Letters in green represent the sequence recognized by the restriction enzymes. Letters in dark represent the nucleotides that do not anneal to the PrP sequence. (B) Multiple cloning site (MCS) of pBI-G vector contains Pst I and Not I restriction sites. Vector was linearized by enzymatic digestion and dephosphorilated. (C) PrP cDNA was subsequently ligated into linearized pBI-G. Dark arrows represent the two minimal CMV promoter driving expression of PrP and lacZ. Tetracycline-Response-Element (TRE) is shown in maroon.

Half ligation mixture was used to transform 50 μ l of Max efficiency DH5 α competent cells (Invitrogen Inc., Carlsband, CA), which have a transfection efficiency of 10^7 . The transformation mixture was spread on YT agar plates with 100 μ g/ml of ampicillin (Roche Diagnostics, Mannheim, Germany) and grown at 37°C for 18 h. 10 colonies selected for ampicillin resistance were isolated from the plate using a sterile pipette tip and used to inoculate 5 ml of YT with 100 μ g/ml of ampicillin. After 10 h, bacteria from 2 ml of culture were harvested by centrifugation at 10,000 x *g* for 5 min in a microcentrifuge (Biofuge, Heraeus, Hanau, Germany). After pouring off the supernatant, plasmid DNA was isolated from cell pellet using the Wizard Plus SV Minipreps DNA Purification System (Promega Corporation, Madison, Wisconsin). Half of the total DNA sample (25 μ l) was digested with 0.5 μ l of Pst I and 0.5 μ l of Not I in a final volume of 30 μ l for 2 h at 37°C to release insert DNA from the plasmid. To screen for the presence of the insert 10 μ l of digestion product were loaded on 1% agarose.

Two colonies for each construct were chosen based on the presence of the insert and the absence of abnormal products of digestion. 2 ml of the corresponding starting liquid culture was used to inoculate 250 ml of fresh selective YT medium and grown at 37°C with vigorous shaking (300 rpm). After 18 h, 0.85 ml of bacterial culture was added to a labelled tube containing 0.15 ml of sterile glycerol, vortexed and transferred to -70°C for long-term storage. Plasmid DNA was purified using a QIAfilter Plasmid Maxi kit (Qiagen Inc., Valencia, California) from 200 ml of bacterial culture. To check the plasmid preparation 1 μ g of purified DNA was digested with 2 U of Pst I and 2 U of Not I in a final volume of 30 μ l for 2 h at 37°C. 0.5 μ g of digested plasmid was loaded on 1% agarose gel.

cDNAs were sequenced with the ABI Prism Dye Terminator Cycle ready reaction Kit (Perkin Elmer Inc., Wallesley, Massachusetts). The sequencing reaction mix contained 500 ng of plasmid and 6.4 pmol of primer. Primers were designed following two criteria: 1) percentage of CG between 40 and 60%; 2) length between 17 and 25 nucleotides; 3) T_m between 39° and 45°C. Primers were: 5'-TTAGTGAACCGTCAGATC-3' and 5'-ACTGCATTCTAGTTGTGG-3' spanning respectively nucleotides 85-105 and 7745-7762 of the pBI-G vector, oligonucleotide 5'-TGCCAAAATGGATCATGG-3' spanning residues 407-424 of wild-type PrP cDNA.

2.2 Cell culture

2.2.1 PC12 Tet-on culture

PC12 Tet-on cells, expressing the rtTA were kindly donated by Dr Jornvall, Karolinska Institute, Sweden. Cells were grown in Dulbecco's modified Eagle's medium (see appendix) at 37°C, in a 5% CO₂ atmosphere, on dishes (BD Falcon, Franklin Lakes, NJ) coated with poly-L-lysine (Sigma Aldrich Co., St. Louis, Missouri). They were routinely split by trypsinization to reduce the formation of cell clumps. To split cells, medium was removed from confluent dishes. After washing with phosphate buffer saline (PBS, Invitrogen, Inc., Carlsband, California), to remove any trace of serum (that inhibits trypsin), cell were incubated with a minimal volume of trypsin/EDTA solution (500 µl in 60-mm dishes, 1 ml in 100-mm dishes or 75 cm² flasks) for 3 min at 37°C. Trypsin was inhibited by adding 5-10 ml of fresh medium and cells were seeded in the appropriate dishes. When necessary, after having inhibited trypsin, cells were counted in a Burker chamber. To this aim, 10 µl of cell suspension were

pipetted in the chamber and counted; the average was calculated on the count of three independent squares.

2.2.2 Cell freezing and thawing

Cells were seeded on 100-mm poly-L-lysine coated plates and left grown until confluence was reached. Cells were subsequently washed, trypsinized (as described above) and resuspended in 10 ml of regular medium. Cell suspension was transferred in a 15-ml sterile tube and centrifuged at $1,000 \times g$ for 5 min in a 5804R centrifuge (Eppendorf AG, Hamburg, Germany), equipped with a A-4-81 rotor. Pellet was resuspended in 5 ml of cold medium containing 10% dimethyl sulfoxide (DMSO) (see appendix), divided in 5 criovials (Nalge Company, Rochester, New York) and left on ice for 30 min. Vials were subsequently transferred to a criobox (Nalge Company, Rochester, New York) and pre-frozen at -80°C for 8 h before stocking in liquid nitrogen.

To thaw cells, the frozen vial was incubated at 37°C for 1 min. The cell suspension was immediately transferred to a 15-ml tube containing 10 ml of regular medium without selective agents and centrifuged at $1,000 \times g$ for 5 min in a 5804 centrifuge (Eppendorf AG, Hamburg, Germany). Cell pellet was resuspended in 5 ml of medium and cells transferred on a 60-mm dish coated with poly-L-lysine. When necessary, selective agents were added after 18 h.

2.2.3 Test sera for Doxycycline contamination

Three lots of horse serum (HS) were tested for doxycycline (Dox, Sigma Aldrich Co., St. Louis, MO) contamination using the CHO-AA8-Luc control Cell Line (kindly donated by Dr Jornvall, Karolinska Institute, Sweden), expressing

the tTA. CHO were grown in alpha-minimal essential medium (MEM- α) (see appendix) on 60-mm dishes without coating. They were trypsinized, frozen and thawed as described for PC12 cells. To test each lot, 6 aliquots of 0.5×10^5 CHO-AA8-Luc Tet-off cells were plated into 2 ml of complete alpha-minimal essential medium supplemented with 10% HS of the lot to be tested in 6-well culture plates. The inducer (Dox) was added to a final concentration of 10^{-4} , 10^{-2} , 10^{-1} , 10 and 10^2 ng/ml and the cells were grown for 48 h. Each sample was assayed for luciferase activity using the Luciferase Reporter assay Kit (BD Clontech, Palo Alto, California). None of the lots of HS tested showed Dox contamination.

2.2.4 Transfection

2.2.4.1 Transient transfection

PC12 Tet-on cells, stably expressing the reverse controlled transactivator, were plated on a 6-well plate (BD Falcon, Franklin Lakes, NJ) and transfected with LIPOFECTAMINE Plus reagent (Invitrogen Inc., Carlsband, California) when cells reached 80% confluence. 0.4 μ g of pBI-G and 4 μ g of pTeT-tTS, or 4 μ g of pBI-G alone were diluted in a vial containing 200 μ l of OPTIMEM (Invitrogen Inc., Carlsband, California) (vial n°1 and 2, solution A). A third vial contained OPTIMEM, without DNA (vial n°3, solution A). At the same time, three mixtures of solution B were made, by mixing 200 μ l of OPTIMEM with 20 μ l of LIPOFECTAMINE. Solutions A and B were combined, mixed and incubated at room temperature for 15 minutes to allow DNA-liposome complexes to form. During incubation time, regular medium was removed from cells, by rinsing with PBS. At the end of the incubation time 1.6 ml of OPTIMEM

was added to the tube containing the complexes and overlaid onto the rinsed cells. In particular, each mixture was equally divided in two wells (1 ml/well). Cells were incubated for 6 h at 37°C before adding 1 ml of regular medium containing twice the normal concentration of sera. This medium was replaced with fresh complete medium after 18 h. X-gal staining was performed 48 h after transfection.

2.2.4.2 Stable transfection

PC12 Tet-on cells, stably expressing the reverse controlled transactivator, were plated on seven 100-mm dishes and transfected with LIPOFECTAMINE Plus reagent when cells reached 80% confluence. Five vials contained 0.8 µg of pBI-G/PrP (WT or M128V or D177N/Met128 or D177N/Val128 or PG14), 8 µg of pTeT-tTS (encoding for tTS) and 0.1 µg of pTK-Hyg (encoding for hygromycin resistance) in 100 µl of OPTIMEM (solution A) (Figure 2.2). One vial differed for the presence of 0.8 µg of the empty pBI-G (without the insert) and another for the absence of pTK-Hyg plasmid. Seven vials were subsequently prepared, each containing 100 µl of LIPOFECTAMINE diluted in 100 µl of OPTIMEM (solution B). Solution A and B were combined, mixed gently and incubated at room temperature for 15 min before adding 6.7 ml of OPTIMEM. This final solution was dropped on rinsed cells and incubated at 37°C for 6 h in a CO₂ incubator. Following incubation, 7 ml of growth medium containing twice the normal concentration of serum were added. Transfection medium, which did not contain antibiotics nor selective agents, was replaced with fresh regular medium after 18 h. Cells were grown for 4 weeks in the presence of 80 µg/ml hygromycin (Sigma Aldrich Co., St. Louis, Missouri)

added to the culture medium 72 h post transfection. Every two days half of the medium was removed and fresh medium was gently added to the cells.

Individual clones were picked up by pipetting and seeded in a microtiter plate (BD Falcon, Franklin Lakes, New Jersey) coated with poly-L-lysine. At confluence, each clone was split in two wells of a 48-well plate (BD Falcon, Franklin Lakes, New Jersey) (1/3 of the cells in one well and 2/3 in the other). The more concentrated well, which reached the confluence quickly, was induced for 24 h with 1 μ g/ml of Dox and screened for PrP expression by Western blot with anti-PrP antibody 3F4. Clones expressing PrP were progressively expanded in wells with a bigger diameter, while the non-expressing clones were discarded. Each positive clone, grown in a 100 mm dish was frozen. Finally, in order to exclude background expression in the absence of the inducer and to compare the expression level after induction, each clone was treated with 0-1 μ g/ml of Dox and analysed by Western blot. To screen cell transfected with the empty pBI-G, β -galactosidase activity was revealed by X-gal staining (see below) after Dox induction.

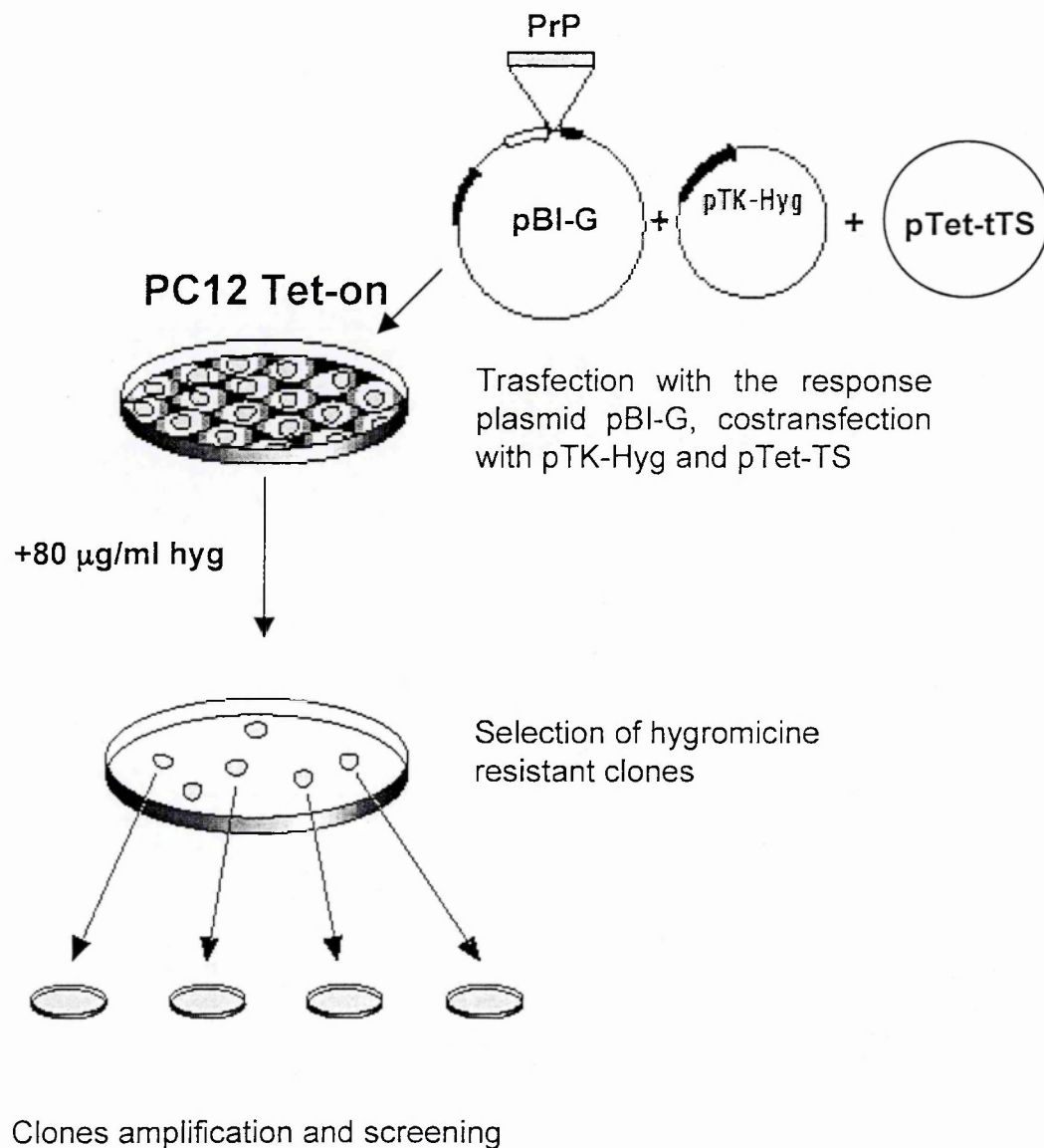


Figure 2.2: Scheme of transfection.

PC12 Tet-on, expressing rtTA, were transfected with pBI-G/PrP, pTK-Hyg, pTet-tTS as described in chapter 2. After selection with 80 µg/ml hygromycin clones were amplified and screened for PrP expression.

2.2.5 Neuronal differentiation

To induce neuronal differentiation, 5,000 PC12 cells/well were seeded on 96-well plate coated with rat tail collagen. The day following plating, the medium was replaced by low serum medium (see appendix) containing 100 ng/ml Nerve Growth Factor (NGF, Harlan Inc., Indianapolis, Indiana) and 200 μ M 8-(4-chlorophenylthio)adenosine3':5'-cyclic monophosphate sodium salt (CPT-cAMP, Roche Diagnostics, Mannheim, Germany). Cells were used after 7 days of NGF treatment, when 80% of cells were differentiated, as judged by neurite outgrowth.

2.2.6 Microglia and PC12 co-culture

Microglia cultures were prepared by dissociating cerebral cortices of newborn rats. Brains from post-natal day 0-5 rats pups were dissected from the skull and meninges removed. Tissue was transferred in a 15-ml tube containing 3 ml of PBS and centrifuged at 1,000 x g for 5 min in a 5804R centrifuge (Eppendorf AG, Hamburg, Germany), equipped with a A-4-81 rotor. PBS was subsequently removed and 2 ml of medium was added. Tissue was disrupted by pipetting and resuspended cells were seeded on poly-L-lysine coated 75-cm² flasks. Cultures were maintained at 37°C with 5% CO₂ for 2 weeks until glia cultures were confluent.

4 x 10⁶ PC12 cells were plated on 100-mm dishes coated with rat-tail collagen (150 μ g/ml in 30% ethanol) and differentiated as described in section 2.2.5. After 7 days cells were washed with PBS, trypsinized and counted. 30,000 cells were diluted in 100 μ l of DMEM containing 10% FBS, 2 mM

glutamine, 5 mM penicillin/streptomycin and 100 ng/ml NGF and seeded on the collagenated 96-well dish. The day after plating PC12 microglial cells were isolated from 75-cm² flasks by shaking cultures at 260 rpm for 18 h. Microglia dislodged into the medium was further purified and counted. 50,000 cells were diluted in DMEM containing 10% FBS, 2 mM glutamine, 5 mM penicillin/streptomycin and 100 ng/ml NGF, and added to PC12 cell cultures.

The day after PC12 alone or co-cultured were incubated with 0-1 µg/ml of Dox for 48 h before proceeding with MTT assay.

2.2.7 Cell viability

Cells were plated in 96-well plate (20,000 c/w) and treated for 24-96 h with 0-3 µg/ml Dox before testing cell viability. Medium was replaced every two days and fresh Dox was added. To test cell viability of NGF-treated PC12 Tet-on cells, 4×10^6 PC12 cells were plated into 100-mm dishes coated with rat-tail collagen (150 µg/ml in 30% ethanol) and differentiated as described in section 2.2.5. After 7 days cells were washed with PBS, trypsinized and counted. 20,000 cells were plated in 96-well plate and treated for 48-96 h with Dox. In order to test the effect of PrP expression under stress conditions, cells were also cultured in the absence of serum or in the presence of hydrogen peroxide (H₂O₂) or tunicamycin (Sigma Aldrich Co., St. Louis, Missouri). In the first case 20,000 cells/well were plated in regular medium, treated with 0-1 µg/ml Dox for 24 h before removing serum. Viability was measured after overnight serum deprivation. Alternatively, cells were plated and induced for 24 h with 0-1 µg/ml Dox before treating with 0-75 µM H₂O₂ or 0-5 µg/ml tunicamycin for 18 h. In each experiments, one well for each condition was lysed in 30 µl of Laemli

buffer 1X and PrP induction was tested by Western Blotting. Cell viability was measured by determining the reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich Co., St. Louis, Missouri) or by staining DNA with crystal violet (Sigma Aldrich Co., St. Louis, MO). 15 μ l of MTT were added to each well containing 150 μ l of medium followed by incubation for 2 h at 37°C in a humidified incubator. 200 μ l of acidic propanol was added to dissolve the blue formazan crystals. The absorbance of the solution was read at 540 nm wavelength using a Labsystem Multiskan (Dasit S.p.A, Cornaredo, Italy). For crystal violet staining cells were washed with PBS and 100 μ l of crystal violet solution was added to each well for 2 minutes. Plates were abundantly washed with H₂O and air-dried. 100 μ l of citrate solution was added to dissolve the crystals. The absorbance of the solution was read at 540 nm wavelength using a Labsystem Multiskan. Viability was expressed as a mean percentage of absorbance of control cells \pm s.d.

2.3 Biochemical analyses

2.3.1 Screening of clones

To assay PrP expression, each clone was seeded in 2 wells of a 48-well dish (BD Falcon, Franklin Lakes, New Jersey), and incubated with or without 1 μ g/ml Dox for 24 h. Each well was washed with PBS and 60 μ l of Laemli sample buffer 1X were added. Samples were heated for 5 min at 95°C, and 25 μ l were analysed by Western blot using antibody 3F4 (1:5,000, see below).

2.3.2 Protein quantification

Protein extracted from cellular lysates were quantified using the BCA Protein assay Reagent Kit (Pierce Inc., Rockford, Illinois). This is a colorimetric method based on the biuretic reaction, i.e. the reduction of Cu^{2+} to Cu^{1+} by proteins. 25 μl of cellular lysate diluted 1:5 and 1:10 in H_2O were pipetted into one microplate well. A fresh set of protein standard was prepared by serially diluting bovine serum albumine (BSA) stock solution (2 mg/ml). 200 μl of reaction mix, prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B, was subsequently added to each well and incubated for 30 min at 37°C . The absorbance of the solution was read at 570 nm wavelength using a Labsystem Multiskan (Dasit).

2.3.3 Detergent insolubility and Proteinase-K (PK) resistance assays

To assay detergent insolubility and PK resistance of PrP, cells were seeded in a 25-cm² flask (BD Falcon, Franklin Lakes, NJ) and treated with 1 $\mu\text{g}/\text{ml}$ Dox when cells reached 90% confluence. After 24 h induction cells were lysed in 1.5 ml of lysis buffer at 4°C for 20 min. Lysates were centrifuged at $14,000 \times g$ in a 5804R Eppendorf centrifuge (Eppendorf AG, Hamburg, Germany) equipped with a F-45-30-11 rotor for 5 min at 4°C and protein concentration of the supernatant was measured with the BCA protein assay, as described. 100 μg were subsequently centrifuged for 40 min at $186,000 \times g$ in OPTIMA MAX-E ultracentrifuge (Beckman Coulter Inc., Fullerton, California) with a TLA-55 rotor to separate the soluble (S) from the insoluble (P) fraction. Soluble proteins were precipitated with 4 volumes of methanol for 2 h at -20°C ,

followed by 30 min at 14,000 x g with a T-60-11 rotor. In this rotor samples are centrifuged horizontally, allowing concentration of the pellet on the bottom of the tube instead of tube wall, thus facilitating recovery of the precipitated proteins. S and P fractions were finally resuspended in 25 µl of Laemli sample buffer 1X and analyzed by Western blot (see below) using the mouse monoclonal anti-PrP antibody 3F4.

To assay PK resistance 300 µg of protein extracts were digested with 0.25-0.5 µg/ml of PK for 30 min at 37°C. Digestion was terminated by addition of phenylmethanesulfonyl fluoride (PMSF, Roche Diagnostics, Mannheim, Germany) to a final concentration of 5 mM. Proteins were methanol-precipitated and resuspended in 25 µl of Laemli sample buffer 1X before Western blot analysis.

2.3.4 Western blot analysis

Proteins were denatured by heating for 5 min at 95°C in Laemli sample buffer 1X, briefly spun in a 5415D microcentrifuge (Eppendorf AG, Hamburg, Germany) and loaded on 12% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gels). Gels were run for 2 h at 100 V in a Mini-PROTEAN® electrophoresis cell (Biorad Laboratories Inc., Hercules, California). Separated proteins were transferred to a polyvinylidene fluoride membrane (PVDF, Millipore Corporation, Bedford, Massachusetts) applying a constant current of 350 mA to a Mini Trans-blot® (Biorad Laboratories Inc., Hercules, California) apparatus for 1 h. Membranes were blocked with 5% non-fat-dry milk (NFDM) in TTBS (see appendix) before incubation with primary antibody. Primary antibodies were diluted in blocking solution and incubated for 1 h at room

temperature or over-night at 4°C. Membranes were washed 3 x 10 min in TTBS to remove unbound antibody, followed by incubation for 1 h at room temperature with IgG peroxidase-conjugated secondary antibodies. Membranes were washed 3 x 10 min in TTBS before detection with Enhanced Chemiluminescence (ECL, Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, England). This system uses horseradish peroxidase (HRP)-conjugated secondary antibodies for luminol-based detection. The oxidation of luminol by the HRP is detected by exposure to autoradiography films.

2.4 RNA analysis

Cells were plated on 60-mm dishes, treated for 24 or 96 h with 0-1 µg/ml of Dox. At the same time, in order to test PrP expression cell were seeded in a 48-well plate, treated with 0-1µg/ml Dox for 24 or 96 h and lysed in 60 µl of Laemli sample buffer 1X. 30 µl were loaded on 12% SDS-gel and blotted with antibody 3F4.

To isolate RNA, cells were chilled on ice and rinsed with cold PBS before adding 1 ml of RNAwiz (Ambion Inc., Austin, Texas). Cell lysates was transferred to a sterile tube and incubated at room temperature for 5 min. After adding 200 µl (0.2 X starting volume) of chloroform, following 10 min incubation, samples were centrifuged at 14,000 x g for 15 min at 4°C in a 5804R Eppendorf centrifuge (Eppendorf AG, Hamburg, Germany) equipped with a F-45-30-11 rotor. The mixtures separated into three phases, the upper containing RNA, the semi-solid interphase containing DNA, and the lower corresponding to the organic phase, containing proteins and lipids. The upper phase was carefully transferred to a clean, sterile tube and diluted with 500 µl (0.5 X starting

volume) of RNase-free water before adding 1 ml (1 X starting volume) of isopropanol. Samples were incubated at room temperature for 10 min and finally centrifuged at 14,000 x *g* for 15 min at 4°C in a with a T-60-11 rotor to pellet RNA directly on the bottom of the tube. RNA was washed with 1 ml of 75% ethanol, vortexed and centrifuged at 14,000 x *g* for 5 min. Supernatant was discarded and RNA was air dried and resuspended in 30 µl of RNase-free water.

To isolate RNA from tissue, half a brain of the entire cerebellum were homogenized respectively in 2.5 ml or 1 ml of RNAwiz using a glass/Teflon homogenizer; 1 ml of sample was processed as described for cultured cells.

2.4.1 Northern blot

2.4.1.1 Probe labelling

Mouse PrP cDNAs (3F4 tagged), hamster CHOP cDNA, mouse BiP cDNA and mouse GADPH (glyceraldehyde-3 phosphate-dehydrogenase) were used as a template for preparation of the probes. Probes were labelled by the Gene Images Random-Prime Labelling module (Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, England). 10 µg of pCDNA3/moPrPWT and pTZ/moCHOP were digested with specific restriction enzymes to release cDNAs. In particular, pBC12/moPrPWT was digested with 20 U of Hind III and Bam HI (Roche Diagnostics, Mannheim, Germany) and pTZ/moCHOP was digested with 20 U of Xba I and Bam HI. Fragments were separated by electrophoresis on 0.5% agarose low-melting gel and purified with ELUTIP minicolumns. BiP and GADPH cDNAs were kindly provided by Professor Sitia (San Raffaele Scientific Institute, Dibat, Milan). 50 ng of denatured DNA were

incubated for 1 h at 37°C with 10 µl of nucleotide mix (dATP, dTTP, fluorescein-dUTP, dCTP and dGTP), 5 µl of primers and 5 U of Klenow DNA polymerase in a final volume of 50 µl (all the reagents were provided by the kit). Reaction was terminated by adding 20 mM ethylenediaminetetraacetic acid (EDTA). In this reaction fluorescein-11-dUTP partially replaces dTTP so that fluorescein-labelled probe is generated. Detection relies on anti-fluorescein antibody conjugated with alkaline phosphatase, thus avoiding the use of radioactive labelling. The enzyme catalyses breakdown of the CDP star substrate (disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclodecan}-4-yl)-1-phenyl phosphate) producing a chemiluminiscent signal. Detection reagents were provided by Gene Image Detection System (Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, England).

2.4.1.2 Electrophoresis and Blotting

20 µg of total RNA was heated for 15 min at 65°C in denaturing buffer, and chilled on ice. Loading buffer was added to the samples before loading on 1.25 % agarose gel containing 6% formaldehyde. Gel was run submerged in running buffer at 4 V/cm. After viewing by ultraviolet illumination to check RNA quality (Ethidium Bromide, which binds nucleic acids is excited by UV light), RNA was transferred on a nylon membrane (Hybond-N+, Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, England) by capillary elution for 18 h and subsequently immobilized by baking the membrane for 2 h at 80°C. After transfer, the blot was rinsed in SSC 2X (sodium chloride sodium citric acid), pre-hybridised for 2 h at 60°C in hybridisation buffer and hybridised for 18 h at 60°C with the fluorescein-labelled probe (1:1,000 in hybridisation

buffer). Probe was removed by washing once with SSC 2X, 0.1% SDS for 15 min at room temperature, once with SSC 1X, 0.1% SDS for 15 min at 65°C, and once with SSC 0.5X, 0.1% SDS for 15 min at 65°C. Membrane was pre-incubated with 5% NFDM in buffer A (blocking buffer) for 2 h at room temperature before incubating with alkaline phosphatase-conjugated anti-fluorescein antibody (1:5,000 in blocking buffer) for 1 h at room temperature. Unbound antibody was removed by rinsing 4 times with buffer A containing 0.3% Tween-20 and once with buffer A without Tween-20. Membrane was finally incubated for 5 min with 3 ml of CDP star and exposed to Hyperfilm-MP film (Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, England).

2.4.2 Reverse transcriptase-polymerase chain reaction (RT-PCR)

200-500 ng of RNA was primed by incubating at 65°C for 5 min with 300 ng oligo(dT) (Promega Corporation, Madison, Wisconsin). First strand cDNA synthesis was carried out for 1 h at 42°C using 4 µl of 5X reverse transcriptase buffer, 0.6 mM dNTP, 10 U of Avian Myeloblastosis Virus (AMV) Reverse Transcriptase and 20 U of Rnasin Ribonuclease inhibitor (Promega Corporation, Madison, Wisconsin) in a final volume of 20 µl. 5 µl of the reaction mixture was subjected to PCR in a reaction mixture (50 µl) composed of 2.5 units Taq polymerase (Promega Corporation, Madison, Wisconsin), 5 µl buffer 10X (500 mM KCl, 100 mM Tris-HCl pH 9, 1% Triton X-100) 0.25 mM dNTP, 1.5 mM MgCl₂, and 300 ng each primer. The primer pairs used for PCR reaction were MXBP1-534: 5'-CCTTGTGGTTGAGAACCAGG-3' (forward) plus MXBP1-804: 5'-CTAGAGGCTTGGTGTATAC-3' (reverse). 30 thermal cycles comprised

94°C for 30 s, 55°C for 30 s and 72°C for 2 min. 10 µl of the PCR products were subjected to electrophoresis on 2% agarose gel.

2.5 Cell imaging

2.5.1 Immunofluorescence

PC12 cells were seeded on poly-L-lysine-coated glass coverslips in 24-well plates at 50% confluence and induced to express PrP by treating them for 24 h with 1 µg/ml of Dox. Alternatively, cells were differentiated as described in section 2.2.5 before proceeding with immunofluorescence. For surface staining of PrP, living cells were incubated with anti-PrP 3F4 antibody (1:500 in OPTIMEM) for 1 h at 4°C, washed with ice-cold PBS and fixed in 4% paraformaldehyde (PFA) for 1 h at 4°C. After washing with PBS, cells were incubated in blocking buffer for 30 min at room temperature and subsequently with Alexa 488(green)-conjugated goat anti-mouse IgG (Molecular probes Inc., Eugene, Oregon) (1:500 in blocking buffer). After 1 h incubation at room temperature cells were rinsed and mounted in FluorSave (Calbiochem Corporation, San Diego, California). To visualise intracellular PrP and other intracellular antigens, cells were washed with PBS and fixed in 4% PFA for 1 h at 4°C. Cells were then rinsed, permeabilized with 0.1% Triton X-100 for 2 min and blocked for 1 h at room temperature with blocking buffer. Primary antibody (in blocking solution) was then incubated for 1 h at room temperature. Cells were rinsed in 0.1% Tween-20/PBS and incubated with Alexa 488(green)- or 546(red)-conjugated secondary antibody before mounting. To perform co-localisation experiments, anti-PrP antibody was first incubated, followed by the

corresponding secondary antibody, before proceeding with organelle markers staining. Cells were viewed on BX61 Olympus Fluorescence microscope equipped with Olympus Fluorview laser confocal scanning system. Digital images were processed using Adobe Photoshop (Adobe Systems, San Jose, California).

2.5.2 Apoptosis Detection

To investigate if mutant PrP expression induced apoptosis, cells were seeded on poly-L-lysine-coated glass coverslips in 24-well plates at 30-40% confluence and induced to express PrP by treating them with 0-1 $\mu\text{g/ml}$ Dox. After 24-96 h propidium iodide staining or tdT-mediated dUTP-X nick end labelling (TUNEL staining) were performed.

2.5.2.1 Staining with propidium iodide (PI)

Propidium iodide (PI) is a fluorescent stain for nucleic acid, that allow detection of apoptotic cells by morphological analysis of nuclei that appear fragmented and pyknotic.

After PrP induction, cells were washed with PBS and fixed in methanol/acetic acid (3:1) for 5 min at 4°C. Cells were subsequently rinsed and a mixture containing PI was added for 30 min and rinsed again. After mounting, slides were viewed on BX61 Olympus Fluorescence microscope equipped with Olympus Fluorview laser confocal scanning system.

PI staining was also coupled with PrP immunostaining. In this case cells were fixed in methanol/acetic acid (3:1) for 5 min at 4°C, washed and subsequently incubated with antibody 3F4 without previous treatment with

Triton X-100 (this fixation procedure permeabilises cell membranes). Cells were subsequently treated as previously described (see section 2.5.1).

2.5.2.2 TUNEL staining

Because in apoptotic cells DNA is nicked, terminal deoxynucleotidyl transferase (tdT) was used to catalyse the addition of deoxyuridinetriphosphate (dUTP) conjugated with biotin to the 3'-OH terminus of DNA molecules. Apoptotic nuclei were subsequently detected using fluorochrome-labelled avidin.

After PrP induction cells were washed briefly with PBS and fixed in 4% PFA for 1 h at 4°C. Cells were then rinsed and incubated for 30 min in 300 µl of saponin solution. Cells were washed 5 times in PBS (2 min each wash) and incubated in tdT reaction mixture for 1 h in a moist chamber at 37°C. After rinsing 3 times in PBS, cells were blocked in blocking buffer and subsequently incubated with avidin-488 (Molecular probes Inc., Eugene, Oregon) (1:500 in staining buffer) respectively for 30 min and 1 h at room temperature. Cells were washed, mounted and viewed on BX61 Olympus Fluorescence microscope equipped with Olympus Fluorview laser confocal scanning system.

TUNEL staining was also coupled with 3F4 staining. In this case, internal staining for PrP was performed as described in section 2.5.1. After viewing PrP staining, cells were directly incubated with tdT reaction mixture, without previous saponin treatment. The experiment was subsequently performed as described above.

2.5.3 β -galactosidase activity assay

Expression of β -galactosidase is indirectly evaluated by testing its enzymatic activity. If β -galactosidase is expressed, in the presence of the chromogenic substrate 5-bromo-4chloro-3indolyl- β -d-galactoside (X-gal) blue cells are detectable.

Cells were seeded in 24-well plates and induced with 1 μ g/ml Dox for 24 h. Cells were subsequently fixed in 2% paraformaldehyde, 0.2% glutaraldehyde for 5 min at room temperature and rinsed with PBS before adding 500 μ l of X-gal mixture. Samples were viewed after 2-8 h of incubation at 37°C.

Chapter 3: Results

3.1 Development of PC12-Tet on cells expressing WT and mutant PrPs

3.1.1 Generation of constructs encoding PrP from the Tet-on promoter

In order to facilitate the detection of transfected protein, the 3F4 epitope tag was introduced in mouse PrP sequence. The substitutions L108M and V111M corresponding to the 3F4 epitope had been previously introduced into wild-type mouse cDNA or appropriate mouse constructs carrying pathogenic mutations in the laboratory of Dr David Harris, Washington University School of Medicine, St. Louis Missouri. cDNAs encoding 3F4-tagged mouse wild-type PrP (M128, WT), M128V PrP, as well mouse PrP homologues of the human D178N (D177N/M128 and D177N/V128) and nine-octapeptide (PG14) mutations had been cloned into Hind III and Bam HI restriction sites of pCDNA3 vector. In order to sub-clone these cDNAs into pBI-G vector, harbouring the tetracycline-responsive element, Pst I and Not I restriction sites were inserted respectively to the 5' and 3' of the PrP ORF by PCR. Five different PCR reactions were run, using as a template one of each of the different cDNAs cloned in pCDNA3. When 2 μ l of PCR product were run on a 1% agarose gel, a band of approximately 800 bp was detected for the WT construct and the constructs carrying the point mutations, and of 1000 bp for the PG14 construct (Figure 3.1). These corresponded to the expected lengths, suggesting a correct amplification of the bands.

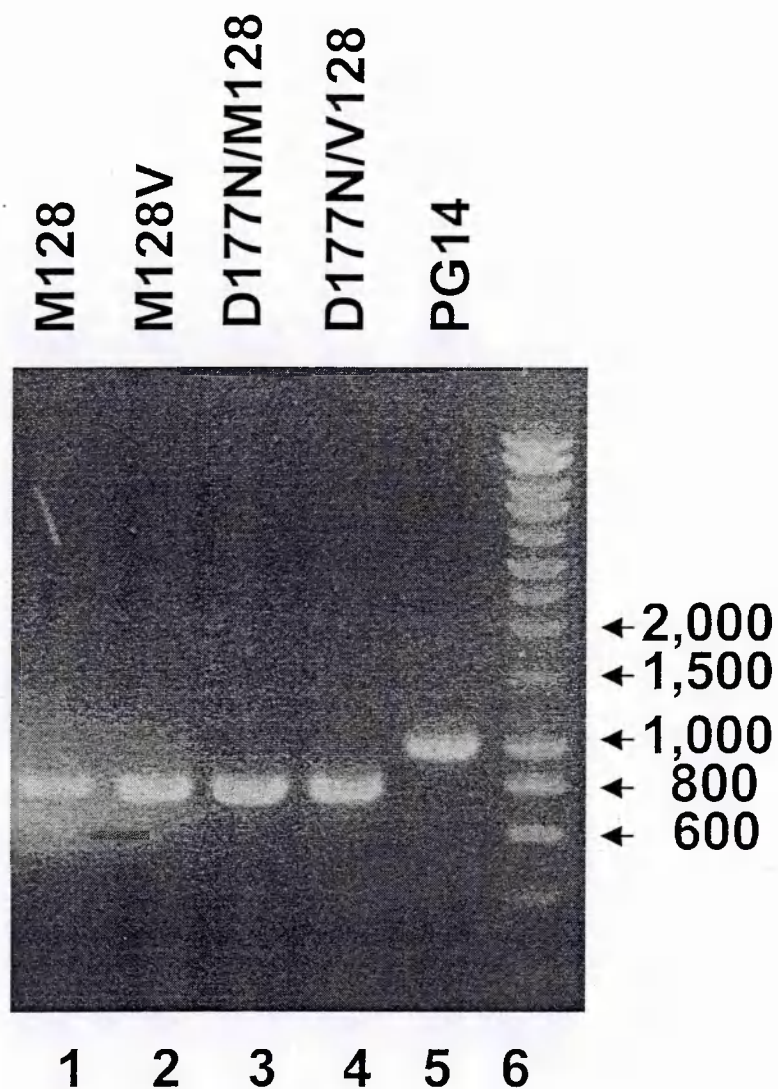


Figure 3.1: Insertion of Pst I and Not I restriction sites respectively to 5' and 3' of PrP cDNAs.

5 different PCR reactions were performed using M128, M128V, D177N/M128, D177N/V128 or PG14 cDNAs cloned in pCDNA3 as a template and primers containing the Pst I and Not I restriction sites. 2 μ l of PCR products were run on 1% agarose gel (lanes 1-5). DNA molecular weight markers (HyperLadder, Bioline) are given in bp (lane 6).

PCR products were digested with Pst I and Not I; pBI-G vector was digested with the same enzymes and the 5' phosphates were removed from both termini by alkaline phosphatase to minimize the possibility that the plasmid recircularized during the ligation reaction.

Ligation of the cDNA insert to the plasmid involves the formation of new bonds between phosphate residues at the 5' termini of double-stranded DNA and adjacent 3'-hydroxyl moieties. For each construct a different ligation reactions was prepared: reaction 1 contained cDNA plus plasmid pBI-G digested and dephosphorylated; reaction 2 contained pBI-G digested and dephosphorylated alone; reaction 3 contained 100 pg of circular pBI-G; and reaction 4 contained no DNA. *Escherichia Coli* competent cells (DH5 α strain) were subsequently transformed with each reaction mixtures, incubated at 37°C in liquid medium to allow expression of ampicillin resistance and subsequently plated on ampicillin (100 μ g/ml)-containing agar plates. Plates were examined after overnight incubation at 37°C: 20-30 colonies were recovered in plate 1, containing cells transformed with both insert DNA and plasmid; 1-2 colonies grew on plate 2 containing cells transformed with restriction digested/dephosphorylated pBI-G; 400-500 colonies grew on plate 3, containing cells transformed with circular pBI-G and no colonies grew on plate 4. Table III.I summarises the transformation experiment performed for each construct and the results obtained.

Table III.I: Schematic representation of ligation and transformation experiment

Plate	Transformed DNA	N° of colonies recovered
1	500 ng insert DNA, 100 ng pBI-G	20-30
2	100 ng digested/dephosphorilated. pBI-G	1-2
3	100 pg circular pBI-G	400-500
4	-	-

These results demonstrated that both ligation and transformation experiments had been achieved. The result of plate 2 indicated that a small percentage of the vector was able to recirculate, suggesting that a few of the colonies recovered from plate 1 could contain pBI-G without the insert.

To identify bacterial colonies containing recombinant plasmid, 10 colonies were picked up from plate 1 and grown in 5 ml of YT liquid medium containing ampicillin. Plasmid DNA was extracted from bacteria grown in the liquid cultures, digested with Not I and Pst I and subsequently analysed on 1% agarose gel. On average, in approximately 6/10 colonies, 2 bands were detected, running at ~10,000 bp and 800 bp (or 1000 bp for PG14), corresponding, respectively, to the linearized vector and the excised cDNA insert. In 2/10 colonies only the 10,000 bp band was detected, indicating the absence of the insert, and in 2/10 colonies no plasmid DNA at all was detected. Figure 3.2 shows an example of colony screening. In this case the insert (running at ~800 bp) was detected in 4/8 colonies, while the rest of the colonies (4/8) were transformed with the empty pBI-G. For each construct, 2 colonies containing the recombinant plasmid were frozen at -80°C in 15% glycerol.

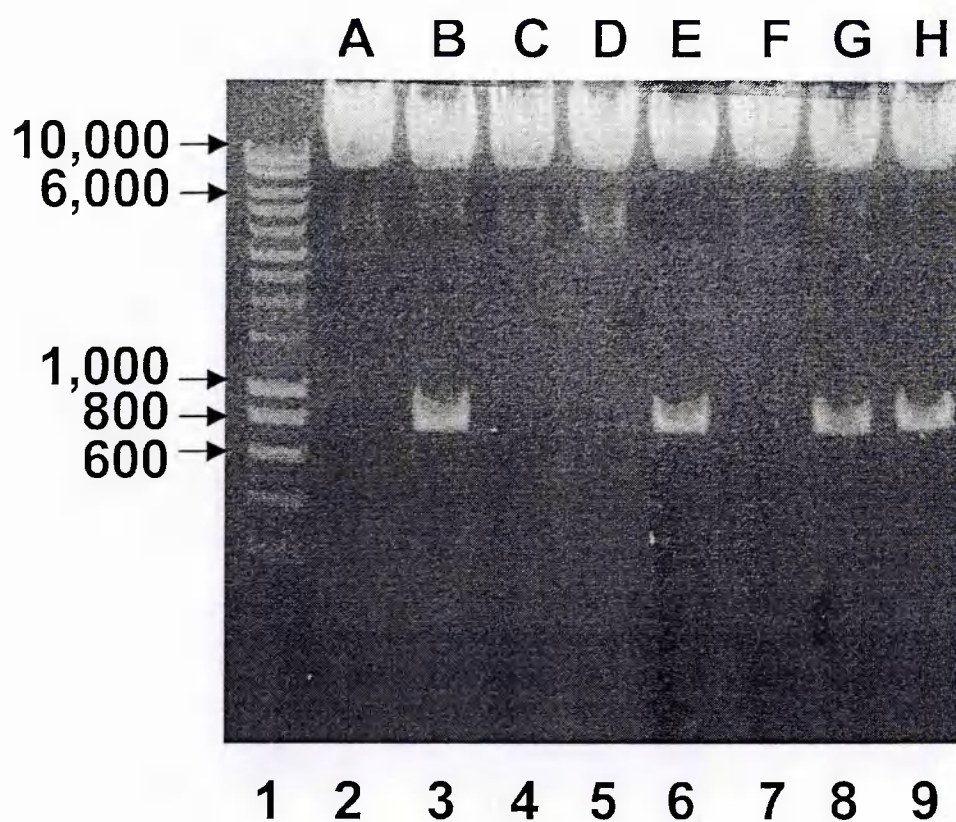


Figure 3.2: Screening of bacterial clones.

In order to screen bacterial cells for the presence of the insert, plasmid DNA was extracted from different clones (clones A-H, lanes 2-9) and digested with Pst I and Not I. A band running at ~800 bp states the presence of the insert (lanes 3, 6, 8, 9). DNA molecular weight markers (HyperLadder, Bioline) are given in bp (lane 1).

To exclude the presence of mismatched nucleotides and ensure correct orientation of PrP cDNAs inside the vector, the inserts and the flanking regions of the plasmid extracted from the selected colonies were sequenced. From sequence analysis of the wild-type construct, an insertion of ~300 bp was discovered upstream to the Pst I restriction site, in both the colonies analysed. In particular, downstream from the promoter and upstream of the Pst I restriction site, the first 300 nucleotides of mouse cDNA were erratically inserted. In contrast, the M128V, D177/M128 and D177N/V128 and PG14 constructs were correctly inserted. In order to amplify in pBI-G/WT not only the PrP cDNAs but even the flanking regions of the plasmid, PCR reactions were performed, using primers p(85-105) and p(7745-7762), spanning respectively nucleotides 85-105 and 7745-7762 of the pBI-G vector. The same reaction was run using pBI-G/M128V and pBI-G/D177N as controls. The PCR product amplified from the WT construct revealed the presence of a band running ~300 nucleotide higher than expected (see comparison with the band of the other constructs in Figure 3.3), confirming that an additional fragment of the PrP cDNA sequence was inserted in the final construct.

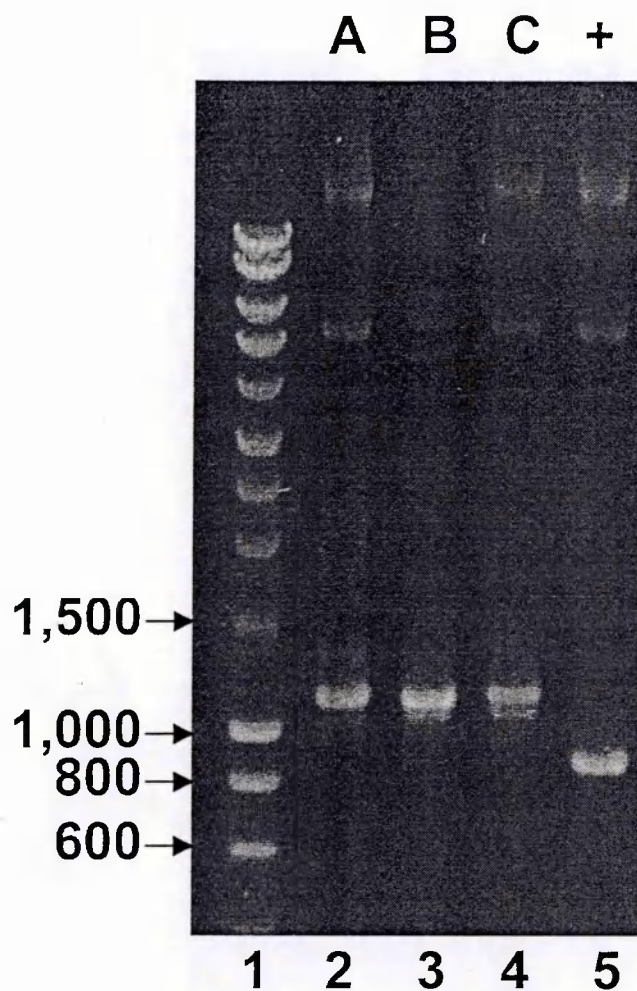


Figure 3.3: Screening of clones by PCR.

Three clones transformed with pBI-G/WT PrP were analyzed for the presence of the 300 bp insertion. Construct purified from each clone was amplified by PCR using primers spanning the vector regions in the 5' and 3' of the cDNA insert (clones A, B, C, lanes 2-4). pBI-G/M128V PrP, previously checked by sequencing, was amplified as a control (5). DNA molecular weight markers (HyperLadder, Bioline) are given in bp (lane 1).

Other colonies were picked up from plate 1 and analysed for the presence of the correct cDNA insert. None of the colonies analysed presented insert DNA with the expected molecular weight. For this reason, ligation of the wild-type PrP cDNA in pBI-G vector and transformation of bacterial cells were repeated as previously described. 5 colonies were picked up from the transformation plate and grown in 5 ml of YT/ampicillin medium. Plasmid DNA was extracted from liquid culture and the presence of the insert was analysed by PCR using primers p(85-105) and p(7745-7762). In all the colonies the amplification gave rise to a band of ~800 bp corresponding to the expected molecular weight. Finally, the absence of mismatched nucleotides and the correct orientation of the cDNA insert were verified by DNA sequencing.

3.1.2 Generation of PC12 Tet-on cells expressing PrP

A major aim of this study was to establish inducible PC12 cell lines using the Tet-on expression system. For this reason we used the bidirectional pBI-G vector which allows coexpression of the gene of interest, PrP, and β -galactosidase. In order to prevent unregulated expression of PrP in the absence of the inducer (Dox), we co-transfected the cells with pTet-tTS, encoding the tet-controlled transcriptional silencer.

Preliminary experiments were conducted before performing the stable transfection. First, susceptibility of PC12 Tet-on cells to the selecting agent hygromycin was tested by incubating the cells with increasing concentration of the antibiotic (0, 10, 20, 25, 50, 80, 100, 150, 200 μ g/ml) for 15 days. Cell treated with 150 and 200 μ g/ml hygromycin died after 24 h, while cells treated with 50-100 μ g/ml of hygromycin died after 2-3 days. A dose level of 10-25

$\mu\text{g/ml}$ was not sufficient to kill cells. Because the optimal concentration should not kill cells immediately, allowing cells that have been transfected to recover from LIPOFECTAMINE toxicity and to express the resistance to the antibiotic, an optimal concentration of 80 $\mu\text{g/ml}$ was chosen for subsequent work.

Transient transfections were then performed in order to test the inducibility of the system. PC12 Tet-on cells were plated in a 6 wells-dish; two wells were transfected with pBI-G and pTet-tTS, two wells with pBI-G alone and two wells were mock transfected. At 24 h post transfection, one well for each treatment condition was treated with 1 $\mu\text{g/ml}$ Dox. Cells were then stained for β -galactosidase activity after 24h of induction. Table III.II summarises this transfection experiment and the results obtained. As expected, β -galactosidase activity was not found in wells (5 and 6) that were subjected to the transfection procedure in the absence of DNA. In cells transfected with pBI-G and pTet-tTS (wells 1, 2) and pBI-G alone (wells 3, 4) the presence of the inducer Dox (wells 2, 4) activated β -gal transcription and subsequent protein translation. In some cells transfected with pBI-G alone, a faint blue staining was detected even in the absence of the inducer (well 3), probably due to low, spontaneous expression of β -galactosidase. Conversely, in the absence of Dox, no signal was detected in cell transfected with both pBI-G and pTet-tTS (well 1), indicating that expression of the tet-controlled transcriptional silencer effectively turned off expression in the absence of inducer. Of note, after induction, staining of cells transfected with pBI-G alone was higher than the one of cells transfected with both plasmids. In particular, not only was the percentage of cells expressing the enzyme higher but also the intensity of the blue signal was greater, indicating that a higher induction efficiency was achieved in the absence of the tet-controlled transcriptional silencer. As the goal was to achieve tightly regulated

expression of PrP and absence of leakage, rather than having high, supraphysiological expression levels, we chose to use the pTet-tTS in combination with pBI-G.

Table III.II: Schematic representation of transient transfection

(-), (+), (++) or (+++) in the fourth column represent the strength of β -gal activity, as indicated by the intensity of the colour in blue cells. (-)= no enzymatic activity, (+)=low activity, (++)= good activity, (+++)=high activity.

Well	DNA	Dox	Percentage of cells expressing β -gal	β -gal – (strength) activity
1	pBI-G, pTet-tTS	-	-	-
2	pBI-G, pTet-tTS	+	20%	++
3	pBI-G	-	1-2%	+
4	pBI-G	+	30%	+++
5	-	-	-	-
6	-	+	-	-

Stable transfection was subsequently performed. pBI-G or pBI-G/PrP plasmids were cotransfected with pTet-tTS and pTK-Hyg as described in the Materials and Methods section. Table III.III summarises the experiment and the results obtained.

Cells in plate 7 died after 1 week of hygromycine selection. Conversely, 4 weeks after transfection, about 100 clones were clearly detectable in plates 1-6. 90 clones of each were picked, expanded and subsequently exposed to 1 μ g/ml Dox for 24 h to induce PrP and β -galactosidase expression. As revealed by Western blot analysis using anti-PrP antibody 3F4, which recognizes transgenically-encoded mouse PrP but not endogenous rat PrP, PrP specific signals were detected after induction in ~5% of clones (Figure 3.4 shows an example of clone screening). Three bands of PrP were detected, corresponding to the un-glycosylated, mono-glycosylated and di-glycosylated forms in all the clones analysed (see also Figure 3.5), indicating that PrP expressed in PC12-Tet-on cells was correctly processed. Few clones showed leakage of PrP expression, as manifested by low PrP expression in the absence of the inducer. Clones that displayed no PrP expression in the absence of Dox, and that had the highest and comparable PrP and β -galactosidase expression level after 24 h of induction were chosen for subsequent experiments. Clones transfected with the empty vector, to be used as a control, were selected based on staining for β -galactosidase activity.

Table III.III: Schematic representation of stable transfection

Dish	PBI-G	PTet-tTS	pTK-Hyg	N° of clones selected	N° of clones analysed	N° of inducible clones	N° of leaky clones
1	Empty vector	Yes	Yes	~100	90	4	0
2	WT PrP	Yes	Yes	~100	90	7	1
3	128V PrP	Yes	Yes	~100	90	6	0
4	D177N(128M) PrP	Yes	Yes	~100	90	5	1
5	D177N(128V) PrP	Yes	Yes	~100	90	5	0
6	PG14 PrP	Yes	Yes	~100	90	4	0
7	Empty vector	Yes	No	0	-	-	-

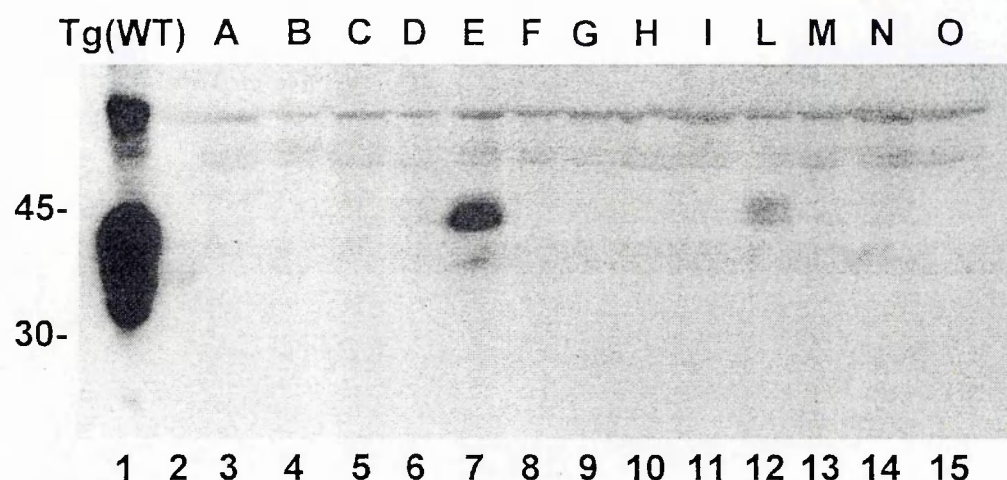


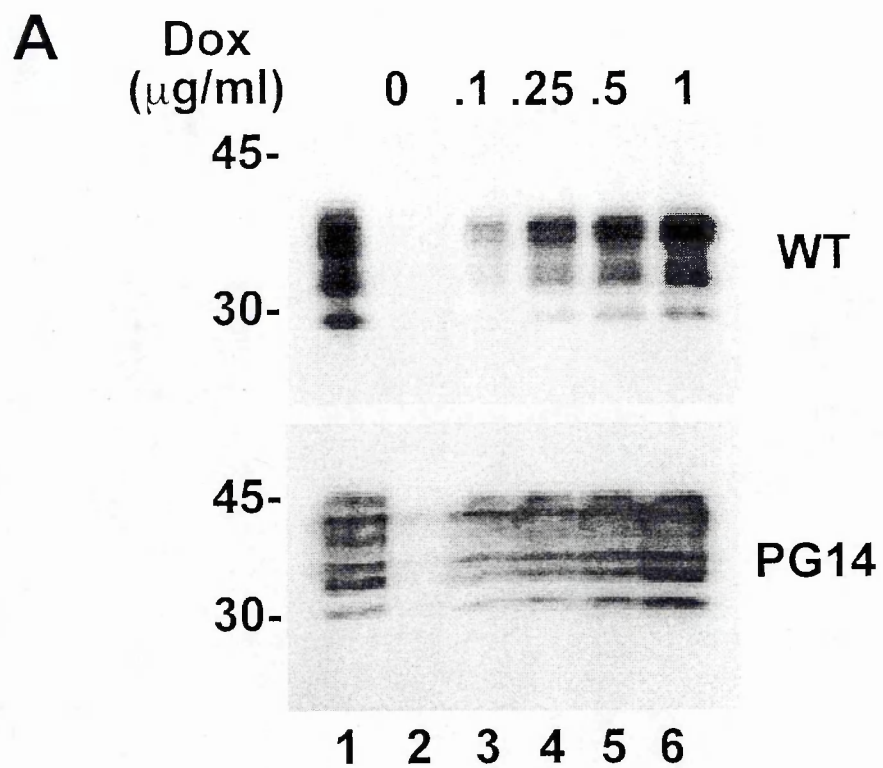
Figure 3.4: Screening of clones selected for hygromycine resistance by Western blot.

Each clone was seeded in one well of a 48-well plate and induced for 24 h with Dox when reached confluency. After lysis samples were analyzed by Western blotting (clones A-O, lanes 3-15). PrP expression was detected only in two clones, E and L (respectively lanes 7 and 12), while the other clones did not respond to Dox treatment. 20 μ g of brain lysate of Tg(WT), expressing 3F4-tagged WT, were loaded as a positive control (lane 1). These mice express 4 fold the endogenous PrP level. Molecular weights are given in kilodaltons, as indicated by Rainbow Markers RNP756(Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, England) loaded in lane 2.

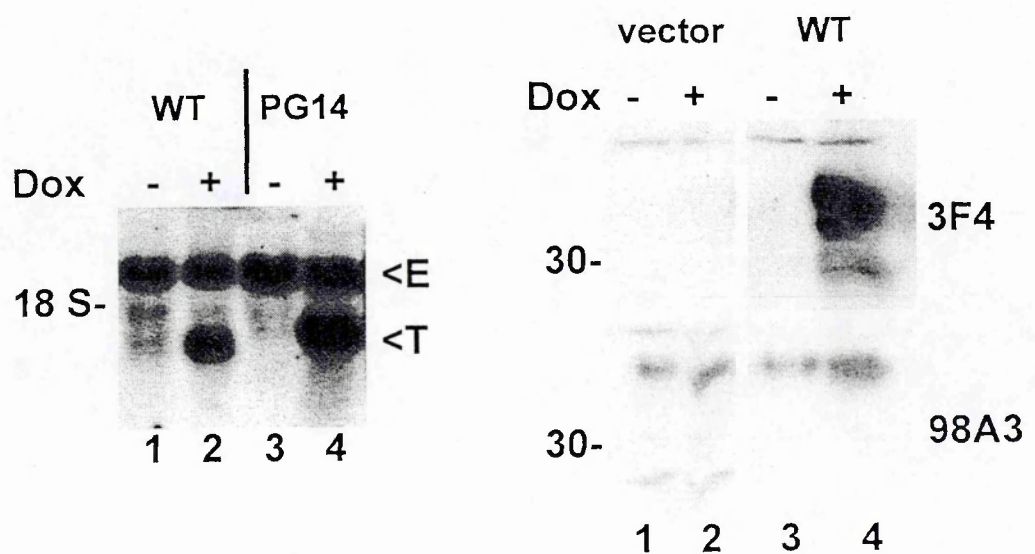
3.1.3 Characterization of PrP expression by PC12 Tet-on cells

The dose-dependency and the kinetics of PrP induction were then analysed by monitoring PrP expression after exposure of cells to increasing concentrations and times of inducer. Cells transfected with wild-type, M128V, D177N/M128, D177N/V128 or PG14 PrP were treated for 24 h with a range of concentrations of Dox (0-2 $\mu\text{g/ml}$) before lysis and Western blot analysis. As shown in Figure 3.5A, PrP expression was tightly regulated: it was undetectable in the absence of the inducer, and increased progressively in a dose-dependent manner within 0.1-0.5 $\mu\text{g/ml}$, reaching a maximum at 1 $\mu\text{g/ml}$ Dox. The expression level of transfected PrP at 1 $\mu\text{g/ml}$ Dox was comparable to that of endogenous PrP gene expression, as revealed by Northern blot and by Western blot with an antibody that recognizes rat and mouse PrP (Figure 3.5B).

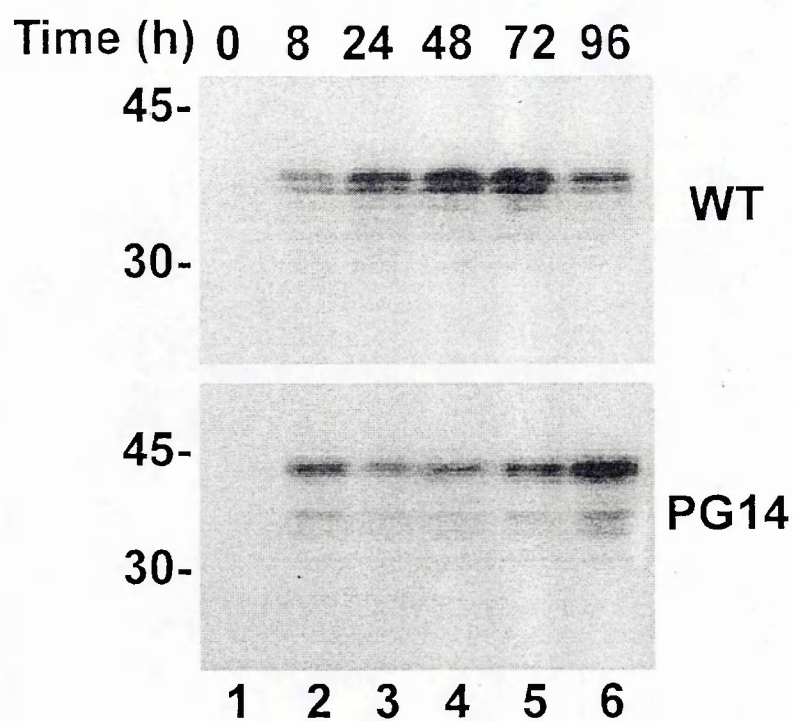
To examine the time-course of PrP expression, cells were harvested at different times after induction with 0.25 $\mu\text{g/ml}$ Dox (Figure 3.5C). PrP expression was detected after 8 h, reached maximum levels at 24-48 h and lasted for at least 96 h. The reversibility of the inducible system was tested by removing/adding Dox (Figure 3.5D). Cells expressing WT PrP were induced for 18 h with 1 $\mu\text{g/ml}$ of Dox (lanes 1-4) and chased in Dox-free medium for 0 h (lane 1), 24 h (lane 2), or 36 h (lanes 3-4) before lysis. After 36 h of chase, cells were re-exposed to Dox for 18 h (lane 4). Cells in lane 5 and 6 were induced respectively for 18 or 72 h. The protein became undetectable after 24-36 h of Dox withdrawal and could be reinduced in a second round of treatment with Dox. Taken together, these results demonstrate that Tet system allowed a tightly regulated PrP expression.



B



C



D

h of induction (1)	18	18	18	18	-	72
h after removal	-	24	36	36	-	-
h of induction (2)	-	-	-	18	18	-

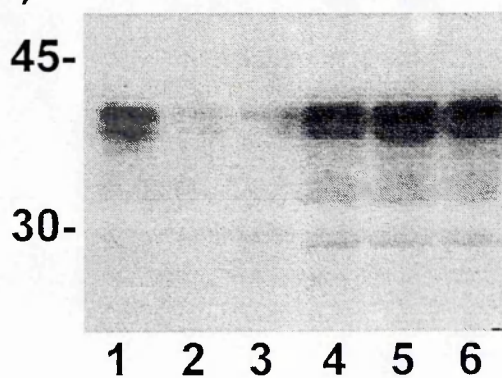


Figure 3.5: Regulation of PrP expression in PC12 Tet-on.

(A) Dose response: cells transfected with WT PrP or carrying the PG14 mutation were induced for 24 h with the indicated doses of Dox and subsequently lysed. 50 μ g of proteins were analysed by Western Blot using the 3F4 antibody. In lane 1, 50 μ g of protein extract of cells constitutively expressing WT PrP (upper panel) or PG14 (lower panel) were loaded as a control.

(B, left) Cells transfected with WT PrP (lanes 1-2) or carrying the PG14 mutation (3-4) were induced for 24 h with 1 μ g /ml of Dox and subsequently lysed in RNA Wiz. 20 μ g of total RNA was analysed by Northern blot using a PrP-specific probe. Both endogenous rat PrP (E) and transfected mouse PrP (T) are detected.

(B, right) Cells transfected with the empty vector (lanes 1-2) or with WT PrP (3-4) were induced with 1 μ g/ml Dox for 24 h (2, 4) or non induced (1, 3). 25 μ g of protein lysates were analysed by Western blotting using 3F4 or 98A3 antibodies.

(C) Time course. Cells expressing WT or PG14 PrP were induced with 0.25 μ g/ml of Dox for the indicated times, lysed and analysed as described in panel A.

(D) Reversibility of the system. Cells transfected with WT PrP were seeded in 48 multi-well plates and induced for 18 h with 1 μ g/ml of Dox (lanes 1-4) and chased in Dox-free medium for 0 h (lane 1), 24 h (lane 2), or 36 h (lanes 3, 4) before lysis. After 36 h of chase, cells were re-exposed to Dox for 18 h (lane 4). Cells in lanes 5 and 6 were induced respectively for 18 of 72 h. PrP expression was analysed by Western Blot using antibody 3F4.

3.2 Characterization of mutant PrP properties

3.2.1 Biochemical properties of PrP molecules

Next, it was tested whether mutant PrPs synthesized in PC12 Tet-on cells acquired the distinctive biochemical properties documented for mutant PrPs constitutively expressed in cultured cells. These properties, which are reminiscent of PrP^{Sc}, include detergent insolubility and proteinase-K resistance.

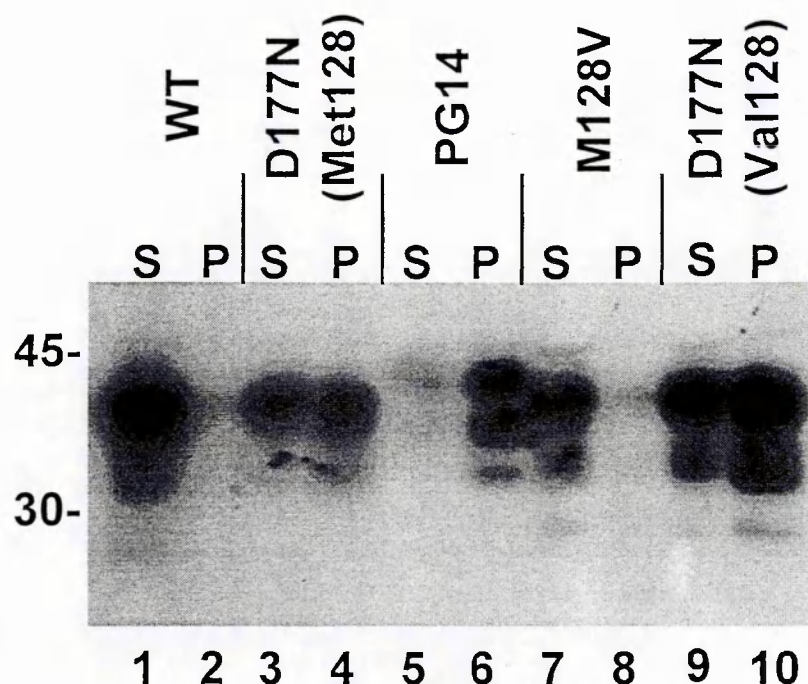
To test detergent insolubility, cells expressing WT, M128V, D177N/M128, D177N/V128 or PG14 PrP were treated for 24 h with 1 μ g/ml Dox. Lysates were subjected to ultracentrifugation at 186,000 $\times g$ for 40 min, a protocol that sediments PrP^{Sc}, but not PrP^C (Caughey et al., 1991a). Supernatant and pellet fractions were subjected to Western blot with antibody 3F4 (Figure 3.6A). While WT PrP was entirely soluble, being recovered exclusively in the supernatant fraction, D177N and PG14 PrPs were partially detergent-insoluble. The two variants of the D177N mutation displayed a similar rate of insolubility, which was lower than for PG14 (D177N/M128: 43 \pm 5%; D177N/V128: 45 \pm 5%; PG14: 72 \pm 8%; mean \pm sd, n=6; p <0.05 (PG14 vs D177N/M128 and D177N/V128) by Student's t-test). Similarly to WT PrP, PrP carrying the Met>Val substitution at codon 128 was entirely soluble.

To assess the protease resistance of PrP, lysates were digested with 0.25 or 0.5 μ g/ml proteinase-K for 30 min at 37°C. As shown in Figure 3.6B, PG14 and D177N moPrPs were cleaved by the protease to yield fragments that migrated between 27 and 30 kDa, the same size as the protease-resistant core of authentic PrP^{Sc}. In contrast, WT moPrP, as well as M128V PrP (not shown in this figure), were completely degraded under these conditions. No substantial

differences were noticed in the degree of protease resistance (evaluated by digestion with different concentration of enzymes) among the different mutants. Finally, the amount of PK-resistant PrP (PK-resistant PrP/total PrP) was difficult to estimate precisely due to the variability of the assay.

These results therefore demonstrated that a portion of mutant PrPs synthesized by the Tet-on system acquires PrP^{Sc}-like properties.

A



B

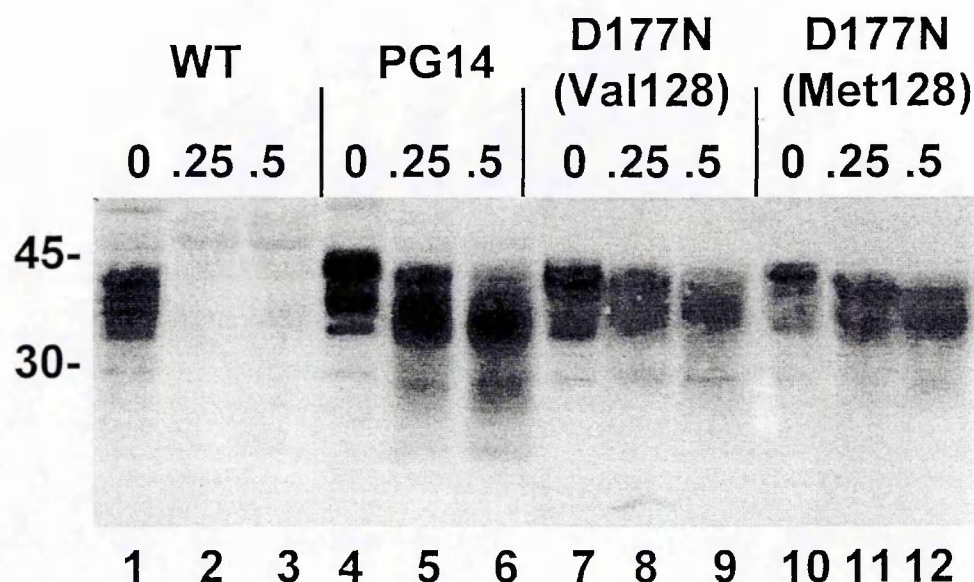


Figure 3.6: Mutant PrP acquires abnormal biochemical properties, typical of PrP^{Sc}.

(A) Cells transfected with PrP, wild-type (lanes 1-2) or carrying the substitution D177N/M128 (3-4), PG14 (5-6), M128V(7-8) or D177N/V128 (9-10), were induced with Dox and subsequently lysed as described in the materials and methods. 100 μ g of protein extract were centrifugated for 40 min at 186,000 \times g to separate the soluble (S) and insoluble (P) fractions. Fractions were analyzed by Western Blot using the antibody 3F4.

(B) Cells transfected with WT PrP (lanes 1-3) or mutant PrPs(4-12) were induced for 24 h with 1 μ g/ml Dox and lysed. 300 μ g of protein extract were digested with 0, 0.25 or 0.5 μ g/ml PK and analysed by Western Blot.

3.2.2 Subcellular localisation of PrP molecules

PrP^C is a cell-surface protein that is attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor at the C-terminus. We first checked the correct localisation of WT PrP in PC12 Tet-on cells. Cells transfected with the empty pBI-G vector or with the pBI-G/moPrP WT plasmid were induced with Dox and stained for PrP with antibody 3F4. To ensure for correct induction, cells were stained for β -galactosidase enzymatic activity, before being immunostained with anti-PrP antibody. As shown in Figure 3.7, β -galactosidase was expressed by both pBI-G and pBI-G/WT PrP cells, while 3F4 staining was detected only in cells transfected with pBI-G/WT PrP.

Several studies have suggested that mutant PrP molecules are inefficiently transported to the cell surface (Capellari et al., 2000; Gu et al., 2003b; Ivanova et al., 2001; Jin et al., 2000; Negro et al., 2001; Petersen et al., 1996; Zanusso et al., 1999). To further investigate these abnormalities the localisation of mutant PrPs in PC12 Tet-on cells was investigated, using confocal microscopy.

To visualize PrP on the cell surface, living cells were stained with anti-PrP antibody 3F4 without permeabilisation (Figure 3.8A, left panels). Despite equivalent levels of PrP induction revealed by Western Blot analysis (Figure 3.8C), cells synthesizing mutant PrP showed much weaker surface staining than those expressing WT PrP. There were noticeable differences among the mutants in the extent of this effect, with PG14 PrP showing less surface staining than D177N/M128 and D177N/V128. No significant differences were noticed between the two variants of the D177N mutation. To visualize intracellular PrP, fixed cells were permeabilised with Triton X-100 prior to application of primary

and secondary antibodies (Figure 3.8A, right panels and Figure 3.8B). It was found that in all cells expressing WT PrP, staining was restricted to the cell surface. In contrast, cells expressing mutant PrPs showed an intense perinuclear staining. Moreover, in 5-10% of these cells, a more widespread intracellular pattern was detectable.

The altered intracellular distribution of PG14 and D177N PrPs in a subset of cells suggested that the transit of the mutant proteins along the secretory pathway was abnormal. To explore the possibility that mutant PrPs were retained in the ER, we double labelled cells with PrP and with calnexin, an ER marker. PC12 Tet-on cells transfected with WT, D177N, or PG14 PrP were induced by treatment with Dox for 24 h. Cells were subsequently fixed, permeabilized and stained with monoclonal antibody 3F4 and rabbit anti-calnexin antibodies followed by Alexa 488 (green)-conjugated anti-mouse and Alexa 546(red)-conjugated anti-rabbit secondary antibodies respectively (Figure 3.9). This double labelling showed no colocalization between WT PrP and calnexin, with the merged image showing no yellow colour. In contrast, mutant PrPs colocalized partially with calnexin, producing a yellow colour. These results indicate that a portion of the mutant PrP resided in the ER. Anti-giantin antibody was then used to label the Golgi apparatus. Cells transfected with D177N and PG14 PrPs were induced with Dox, fixed, permeabilised and stained with rabbit anti-PrP 45-66 and mouse monoclonal anti-giantin antibody followed by Alexa 546(red)-conjugated anti-rabbit and Alexa 488(green)-conjugated anti-mouse secondary antibodies respectively (Figure 3.10). Although some mutant PrP colocalised with giantin, much of the PrP labelling did not. These results suggested that in a subset of cells, PG14 and D177N

PrPs are impaired in their transport to the cell surface and partially retained in the ER.

The distribution of these mutants was also analysed in cerebellar granule neurons (CGN) from Tg mice (Fioriti et al., 2005). In CGN from Tg(WT) mice PrP was mainly found on the cell membrane where it appeared in a patchy distribution along the neurites, consistent with association of the protein with membranal "rafts". In CGN from Tg(PG14) mice, PrP was barely detectable on the cell membrane and along neurites, and was mainly found in intense perinuclear patches, colocalizing with ER and Golgi markers. Low expression on the cell surface and intense intracellular immunofluorescence colocalizing in part with giantin and trap, were observed also in CGN expressing the D177N/M128 and D177N/V128 mutants, although the number of cells displaying this altered intracellular PrP distribution was reduced compared to PG14 neurons.

These data indicate that, similarly to that observed in transfected cells also in primary neurons the pathogenic mutations alter the trafficking of PrP molecules and cause a portion of them to reside abnormally in intracellular compartments.

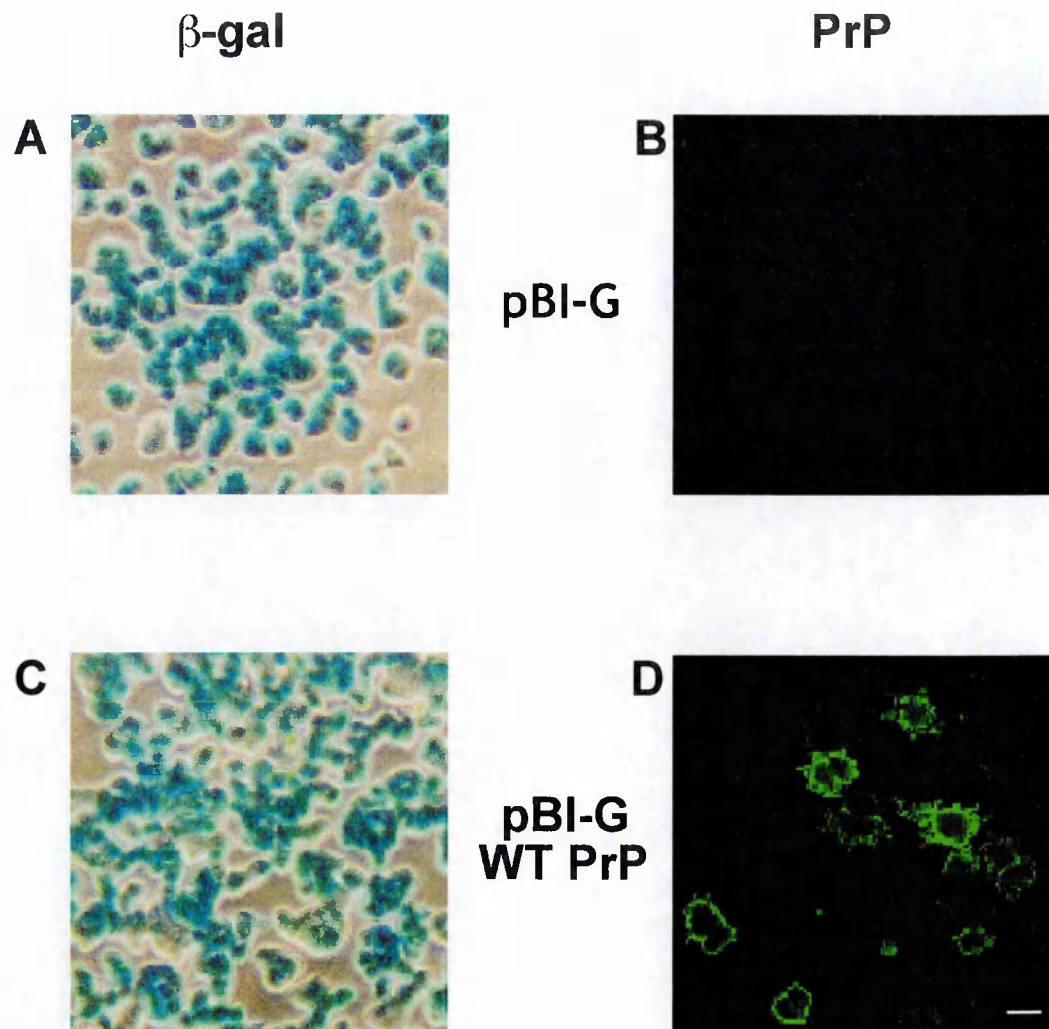


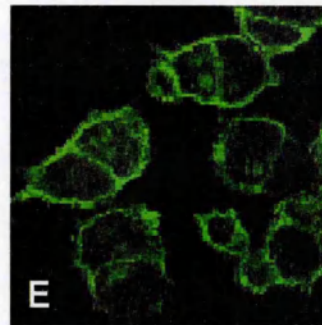
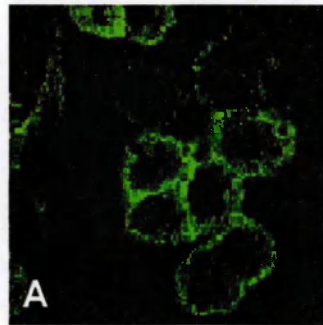
Figure 3.7: The bi-directional promoter allows co-expression of PrP and β -galactosidase.

Cells transfected with the empty pBI-G vector (A, B), or with pBI-G/WT PrP (C and D) were treated for 24 h with 1 μ g/ml of Dox and immunostained for PrP (B, D), or for β -galactosidase activity (A, C). As shown in panel D, PrP is correctly expressed on the surface of cells transfected with pBI-G/WT PrP. Scale Bar, applicable to all panels, is 20 μ m.

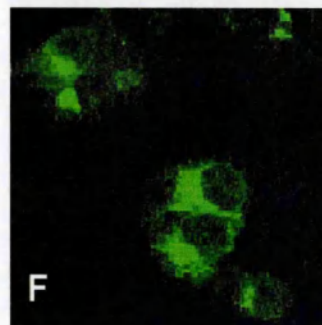
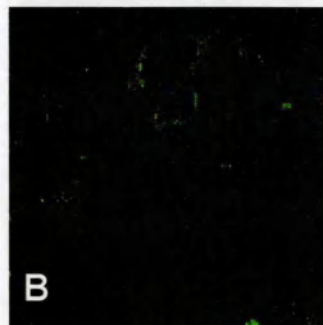
A

Surface PrP

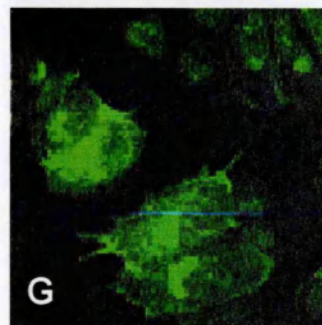
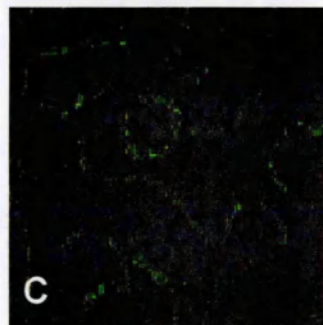
**Intracellular
PrP**



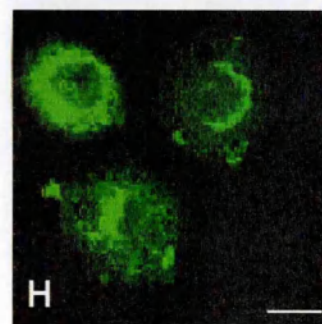
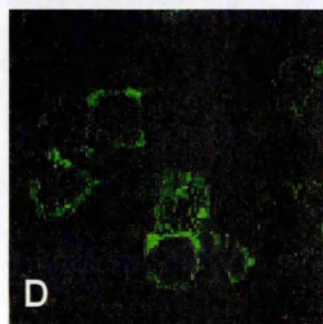
WT



PG14



**D177N
(Met 128)**



**D177N
(Val 128)**

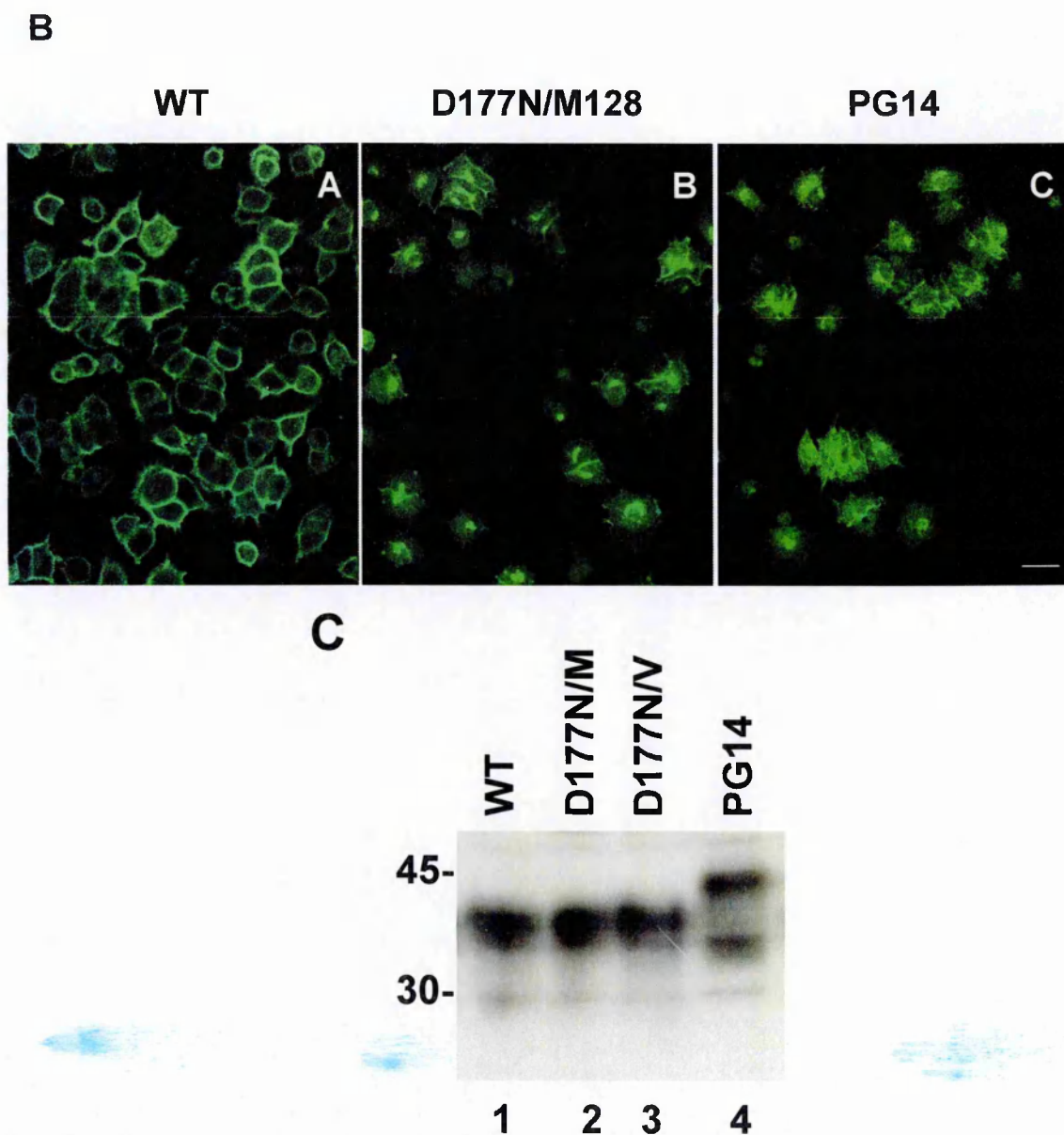


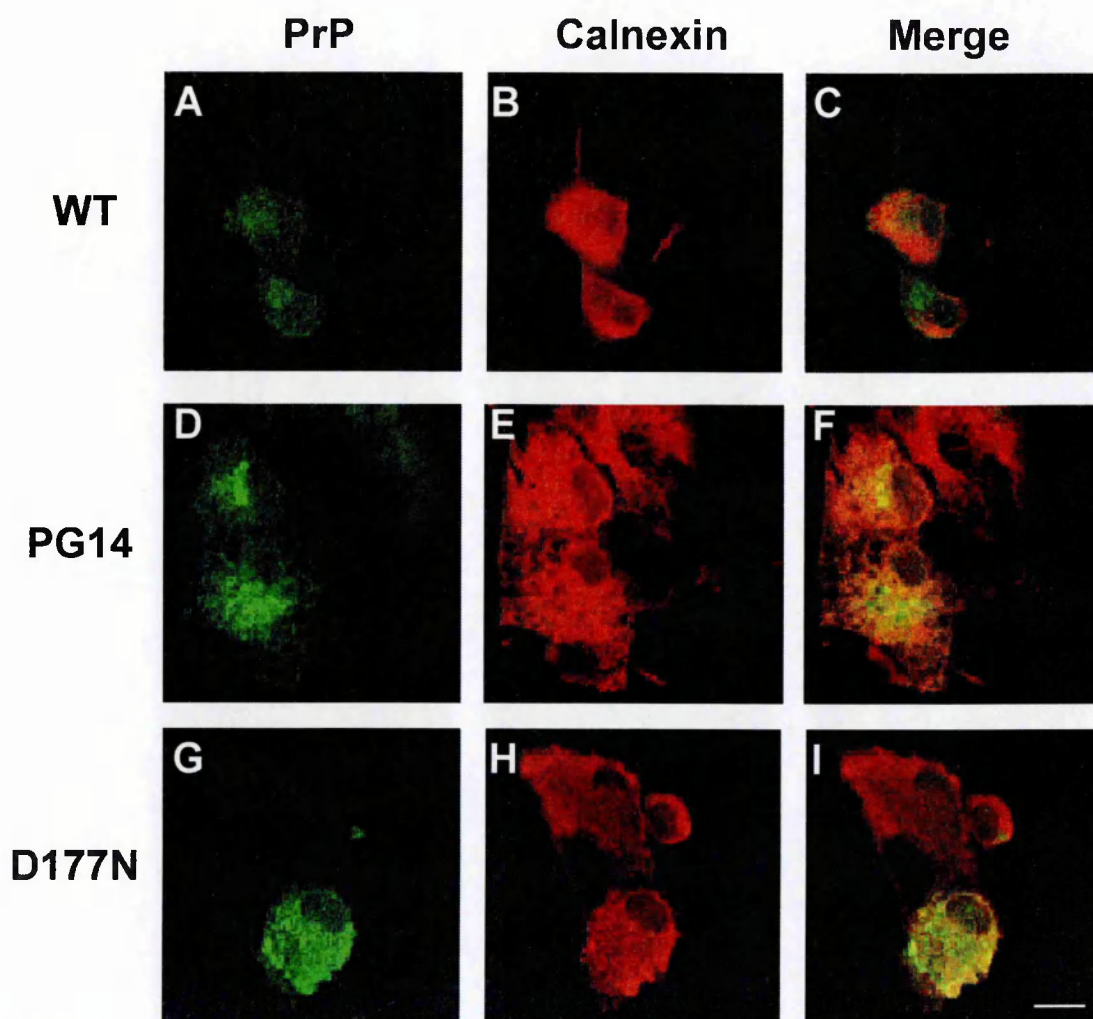
Figure 3.8: Mutant PrPs are less present on cell surface.

(A) Cells expressing WT PrP (A, E), PG14 PrP (B, F), D177N(M128) PrP (C, G) or D177N(V128) PrP (D, H) were incubated with 3F4 antibody without permeabilization to detect PrP on the cell surface (panels A, B, C, D) or fixed and permeabilised before incubation with 3F4 to allow antibody entrance. Scale Bar, applicable to all panels, is 10 μ m.

(B) Low magnification of cells stained for PrP after permeabilisation. Cells expressing WT PrP (A), D177N/M128 PrP (B) or PG14 PrP (C) are shown. Scale Bar is 20 μ m.

(C) Equivalent PrP induction was checked by Western blotting. Cells transfected with WT (lane 1), D177N/M128 (2), D177N/V128 (3) and PG14 PrP (4) were seeded in one well of a 96-well plate and treated with Dox (simultaneously with cells plated for immunofluorescence) before lysis in 25 μ l of laemli sample buffer and separation onto SDS PAGE.

A



B

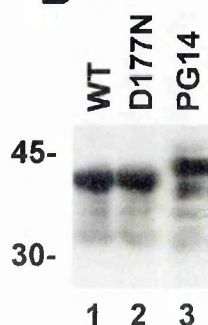


Figure 3.9: Mutant PrPs colocalize with an ER marker.

PC12 Tet-on cells transfected with WT, PG14, D177N/M128 PrPs were differentiated with NGF (100 ng/ml), and PrP expression was induced by treatment with 1 μ g/ml Dox for 24 h. (A) Cells were fixed, permeabilized and stained with mouse anti-PrP antibody 3F4 (A, D and G) and rabbit anti-calnexin antibody (B, E and H) followed by Alexa 488(green)-conjugated anti-mouse and Alexa 546(red)-conjugated anti-rabbit secondary antibodies. Merged green and red images are shown in C, F and I. Scale Bar, applicable to all panels, is 10 μ m. The mutant D177N/V128 did not behave differently from the D177N/M128 and was not included in this figure. (B) Before performing immunofluorescence PrP expression level was checked by Western blotting using 3F4 antibody, as described in Figure 3.8.

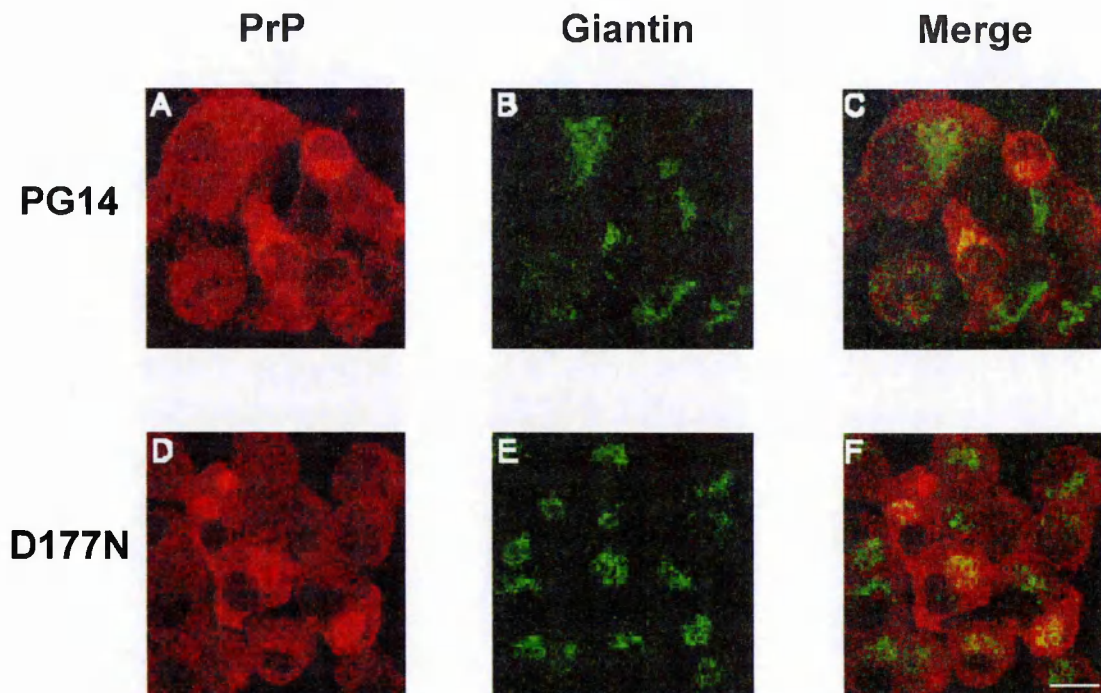


Figure 3.10: Mutant PrPs partially localize with the Golgi marker Giantin.

PC12 Tet-on transfected with pBI-G/PG14 PrP or pBI-G/D177N/M128 PrP were differentiated with NGF (100 ng/ml), and PrP expression was induced by treatment with 1 μ g/ml of Dox for 24 h. Cells were fixed, permeabilized and stained with rabbit anti-PrP antibody P45-66 (A and D) and mouse anti-giantin antibody (B and E) followed by Alexa 546(red)-conjugated anti-rabbit and Alexa 488(green)-conjugated anti-mouse secondary antibodies. Merged green and red images are shown in C and F. Scale Bar, applicable to all panels, is 10 μ m. The mutant D177N/V128 did not behave differently from the D177N/M128 and was not included in this figure.

3.3 Effect of mutant PrP expression

3.3.1 Cell viability after PrP induction

The Tet-on system allowed investigation of the potential cytopathic effect of mutant PrP expression. Transfected cells were maintained in Dox-free medium until the time of each experiment. To quantitatively measure cell viability after PrP expression, cells were seeded in microtiter plates and exposed to different concentration of Dox (0-2 $\mu\text{g/ml}$) for 24, 48, 72 or 96 h. In each experiment, to ensure the same rate of induction, PrP expression level was checked by Western blotting, using the antibody 3F4. Cell viability was tested by the MTT assay and by counting nuclei after staining with propidium iodide or TUNEL. No differences were found between different clones after 24-72 h of induction (see Figure 3.11 at 48 h). However, a decrease in the number of cells expressing D177N and PG14 PrPs was observed after 96 h of Dox exposure (Figure 3.12 and 3.13A). Propidium iodide staining of these cells revealed the presence of fragmented and pyknotic nuclei (Figure 3.12, lower panel D), suggesting that the decrease in viable cell number was due to apoptosis. However, even though the decrease in cell survival was always associated with mutant PrPs and was statistically significant within the same experiments, much variability was noticed between independent experiments with reduction of cell viability detected in only 3 of 10 experiments conducted. Figure 3.13 shows the effect of mutant PrP expression on cell viability: in one case a reduction in cell viability was detected (A), while in the other was not (B).

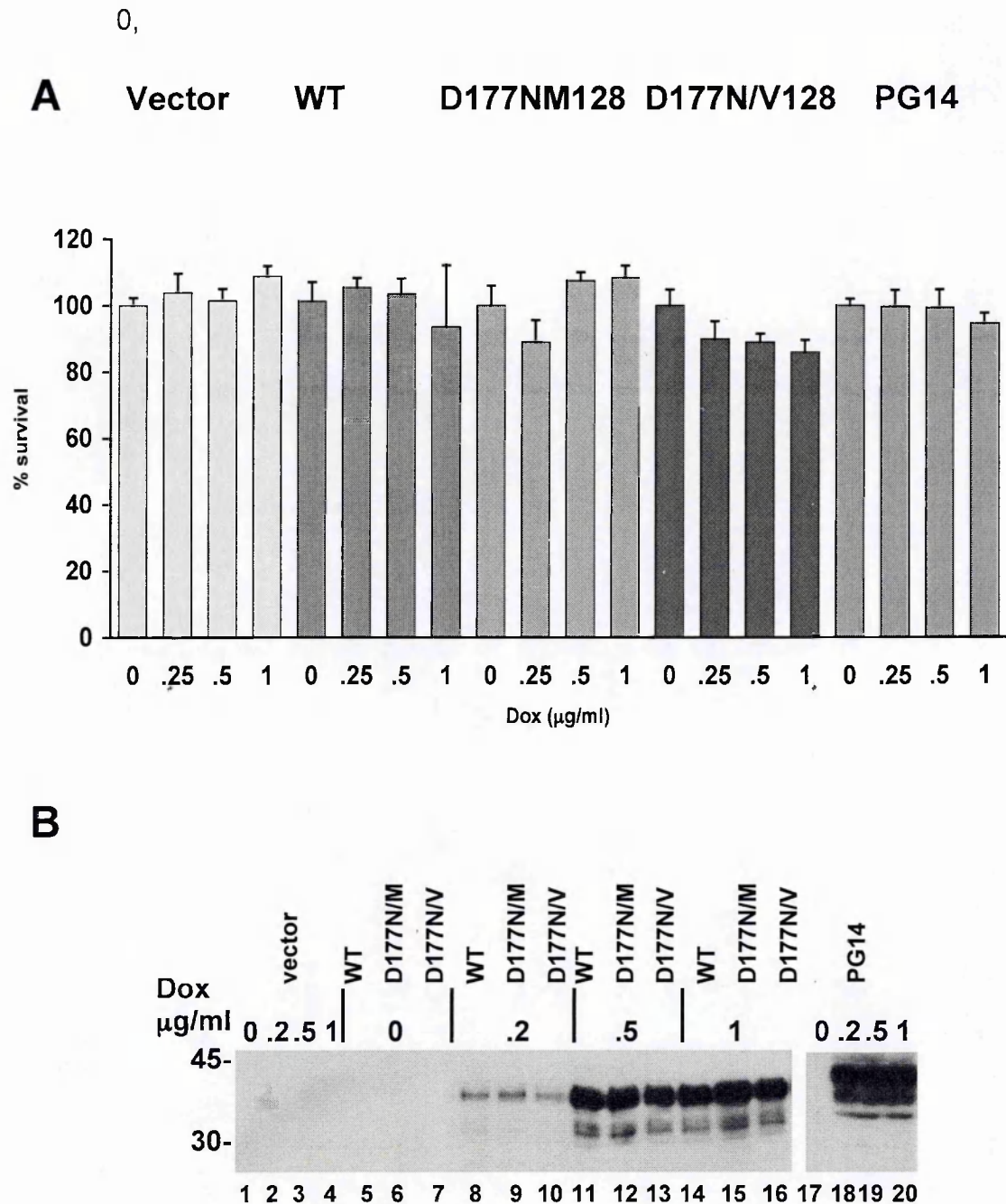


Figure 3.11: Cell viability does not vary after 48 h of Dox exposure.

Cells transfected with the empty vector, WT PrP, D177N/M128, D177N/V128 and PG14 were exposed to different concentration of Dox (0-1 μg/ml) for 48 h. (A) Cell viability was assessed by MTT. Values are the mean±s.d. of 5-8 wells and are expressed as a percentage of control cells (0 Dox). (B) To check PrP induction, cells transfected with the empty vector (lanes 1-4), with WT PrP (5, 8, 11, 14), D177N/M128 PrP(6, 9, 12, 15), D177N/V128 (7, 10, 13, 16) or PG14 PrP (17-20), treated as indicated by the labelling, were analysed by Western blotting. Cells were seeded in one well of a 96-well plate, treated with Dox and lysed in 25 μl of laemli sample buffer before separating on a 12% SDS gel.

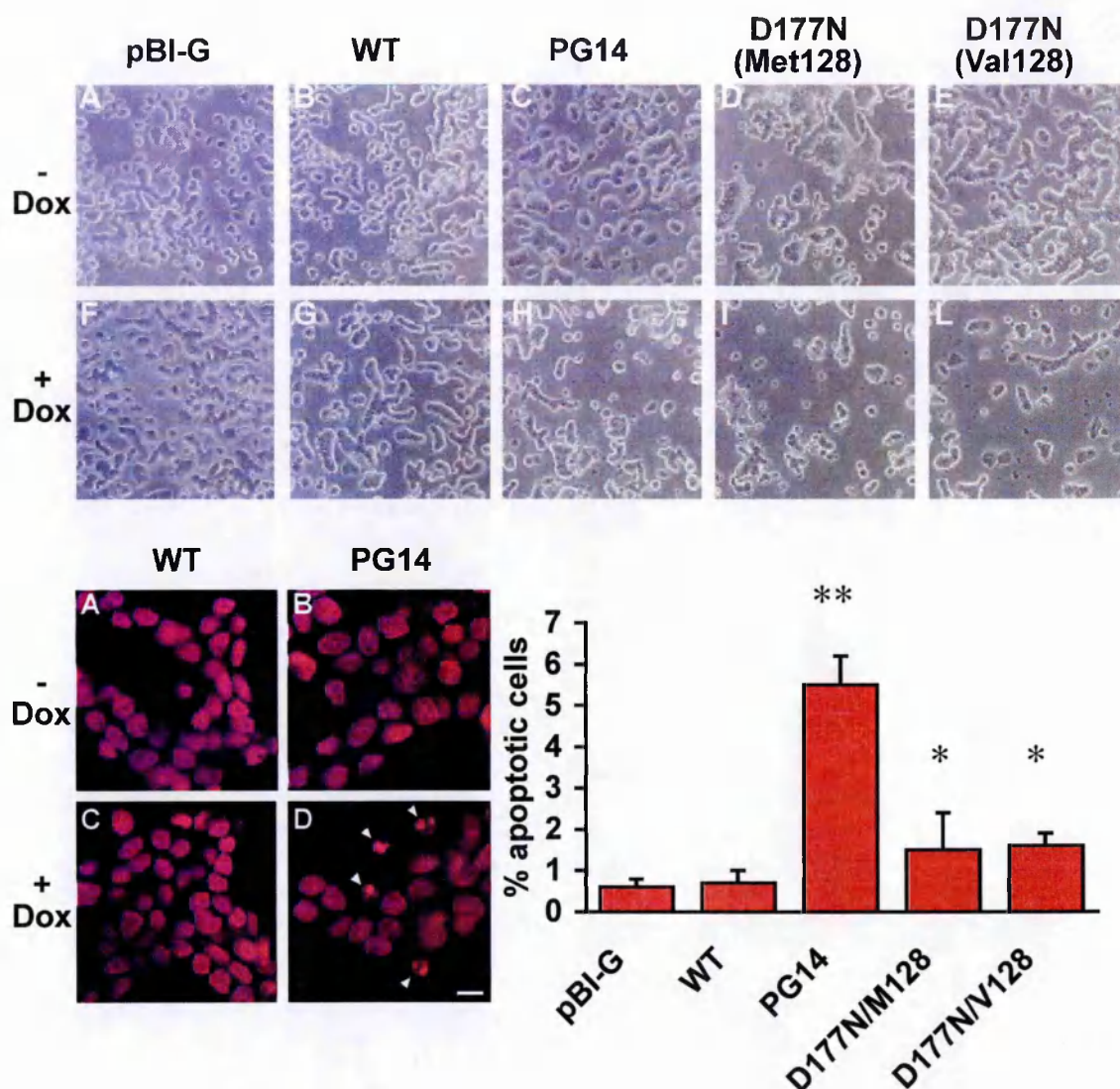


Figure 3.12: Expression of mutant PrP is associated with apoptosis.

(Upper panels) PC12 Tet-on cells transfected with the empty vector (A, F), WT PrP (B, G), PG14 (C, H), D177N(Met128) (D, I) or D177N(Val128) (E, L) were exposed to 0 (A-E) or 1 mg/ml Dox (F-L) for 96 h and examined by phase contrast microscopy. Pictures were performed with a magnification of 40X on a Nikon F-601M camera. PrP expression level after 96 h of induction was checked by Western blotting and was equivalent for all the clones (not shown in this figure).

(Lower panels, left) Cells were subsequently stained with propidium iodide, mounted and analyzed by confocal microscopy. In this panel, only WT PrP (A, C) and PG14 PrP (B, D) are shown before (A, B) and after (C, D) Dox treatment. Pyknotic and fragmented cells expressing PG14 PrP(D) are indicated by arrowheads. Scale Bar is 10 μ M.

(Bar graph, right): Quantitative evaluation of the apoptotic cells after induction of PrP expression. The values are expressed as a percentage of the total number of cells; each bar represents the mean \pm SEM. of 10-12 100 X fields from two independent experiments. * $p < 0.05$ and ** $p < 0.01$ versus cells expressing WT in Student's t-test.

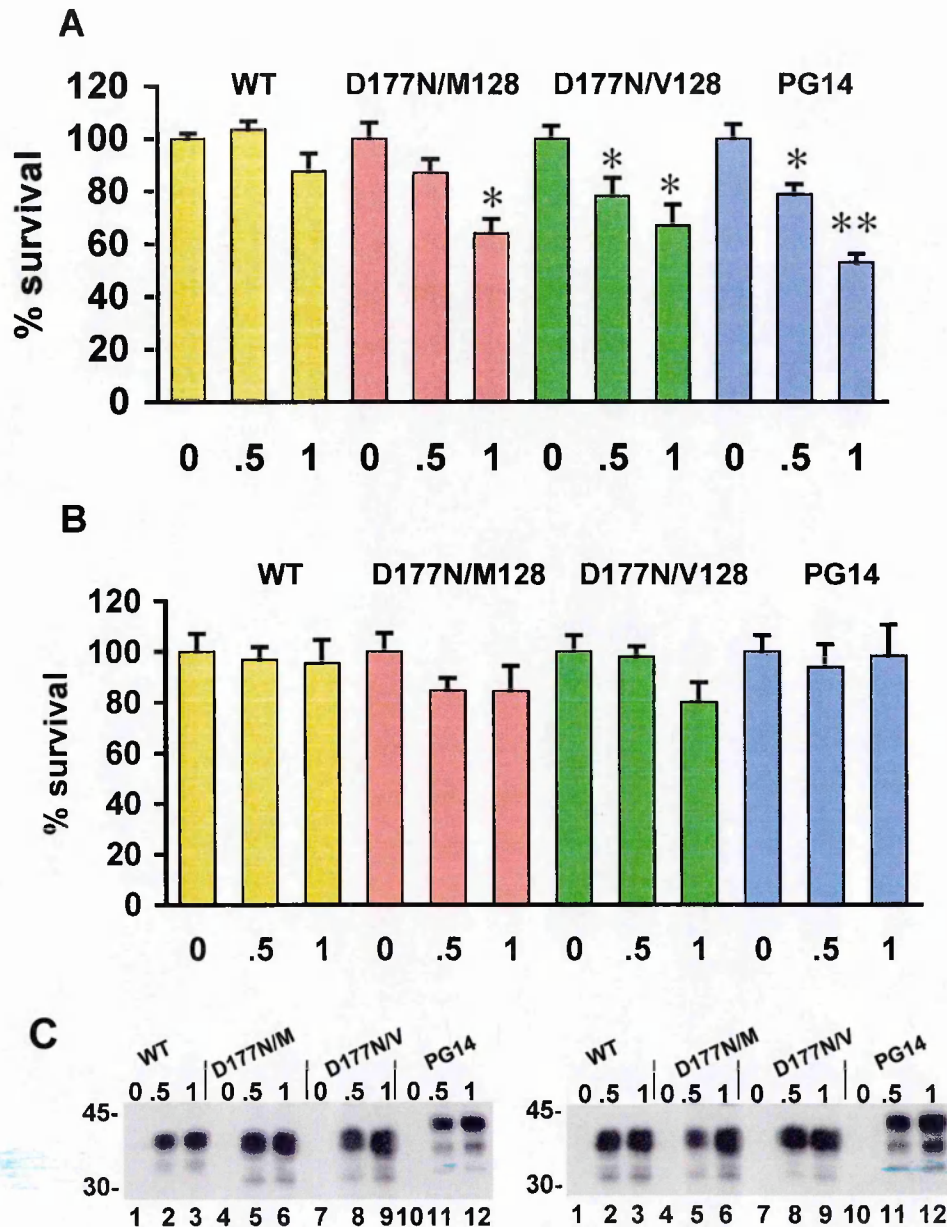


Figure 3.13: The cytotoxic effect of mutant PrP expression is highly variable.

Cells transfected with WT PrP, D177N/M128 PrP, D177N/V128 PrP or PG14 PrP were treated for 96 h with 0, 0.5 or 1 µg/ml Dox. Viability was subsequently assessed by MTT assay, as described in the Materials and Methods. Each value is the mean±s.d. of 5-8 wells expressed as a percentage of the survival of control cells (0 µg/ml Dox). * $p < 0.05$ and ** $p < 0.01$ versus the correspondent dose of cells expressing WT in Student's t-test. While differences in cell viability are statistically significant in A, no differences among the different clones were found in an independent experiment (B). To ensure an equivalent induction in all the clones, PrP expression was analysed by Western blotting. Panel C shows PrP expression level achieved in the experiment A (left) and B (right) for WT (lanes 1-3), D177N/M128 (4-6), D177N/V128 (7-9) and PG14 PrP (10-12). The concentrations of Dox used are indicated above (0, 0.5 or 1 µg/ml).

It was then investigated whether cells undergoing apoptosis were the same cells as those showing an altered intracellular distribution of mutant PrP. To answer this question, cells expressing WT and mutant PrPs were exposed to Dox for 72 h, fixed, permeabilized and immunostained with anti-PrP antibody 3F4 followed by an Alexa 488(green)-conjugated anti-mouse secondary antibody. After immunofluorescence staining, cells were viewed to confirm that PrP was expressed before proceeding with propidium iodide staining. This experiment showed that cells with an altered intracellular PrP distribution displayed a normal nuclear morphology (Figure 3.14) and that none of the apoptotic cells showed retention of transfected PrP in the Golgi/ER.

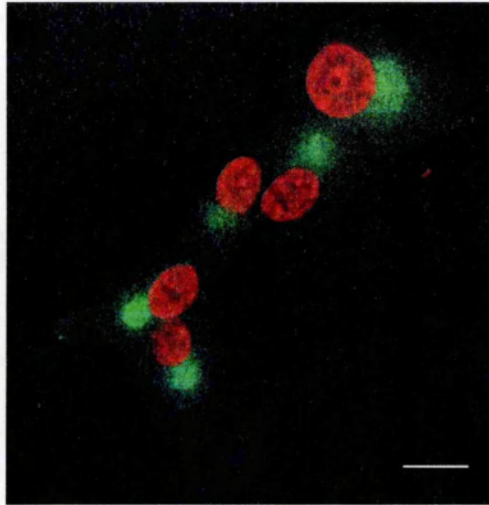


Figure 3.14: Cells with altered intracellular PrP distribution show normal nuclear morphology.

Cells transfected with pBI-G/PG14 PrP were differentiated with NGF and subsequently treated with 1 $\mu\text{g/ml}$ Dox for 72 h before fixing in methanol/acetic acid. Cells were stained with anti-PrP antibody 3F4, followed by Alexa 488(green)-conjugated anti-mouse antibody before performing staining with propidium iodide. PrP staining is shown in green, nuclei are red. Scale Bar is 10 μm .

3.3.1.1 Cell viability after neuronal differentiation

PC12 cells are rat adrenal pheochromocytoma cells that can be differentiated into noradrenergic neurons by treatment with NGF (Greene and Tischler, 1976). Because much variability was noticed in the extent of mutant PrP toxicity (see above), it was investigated whether acquisition of neuronal phenotype resulted in increased susceptibility to mutant PrP expression. Clones transfected with WT, D177N, PG14 PrP or with the empty vector were differentiated with NGF before inducing expression of the PrP protein. After 3-5 days of exposure to NGF, cells started to develop neuron-like processes. The number and length of such processes continued to increase over the next 7-10 days of treatment, until ~80% of the cells in the cultures had ceased dividing and had developed neurites. No differences in the time course or extent of neurite extension was noticed among the different clones. Moreover, the response of these clones to NGF was comparable to the response of parental PC12 Tet-on cells (Figure 3.15A). For all the clones, an equivalent expression level of transgenic PrP after Dox treatment was checked by Western blotting, using the 3F4 antibody (Figure 3.15B). No changes in transgenic PrP expression were detected after NGF treatment (not shown); conversely, changes in the expression level of endogenous PrP due to NGF treatment were not evaluated.

The extent of cell death associated with mutant PrP expression was not significantly altered by differentiation with NGF (when compared with undifferentiated cells transfected with the same PrP construct), demonstrating that acquisition of the neuronal phenotype did not render PC12 cells more susceptible to mutant PrP toxicity (Figure 3.15C).

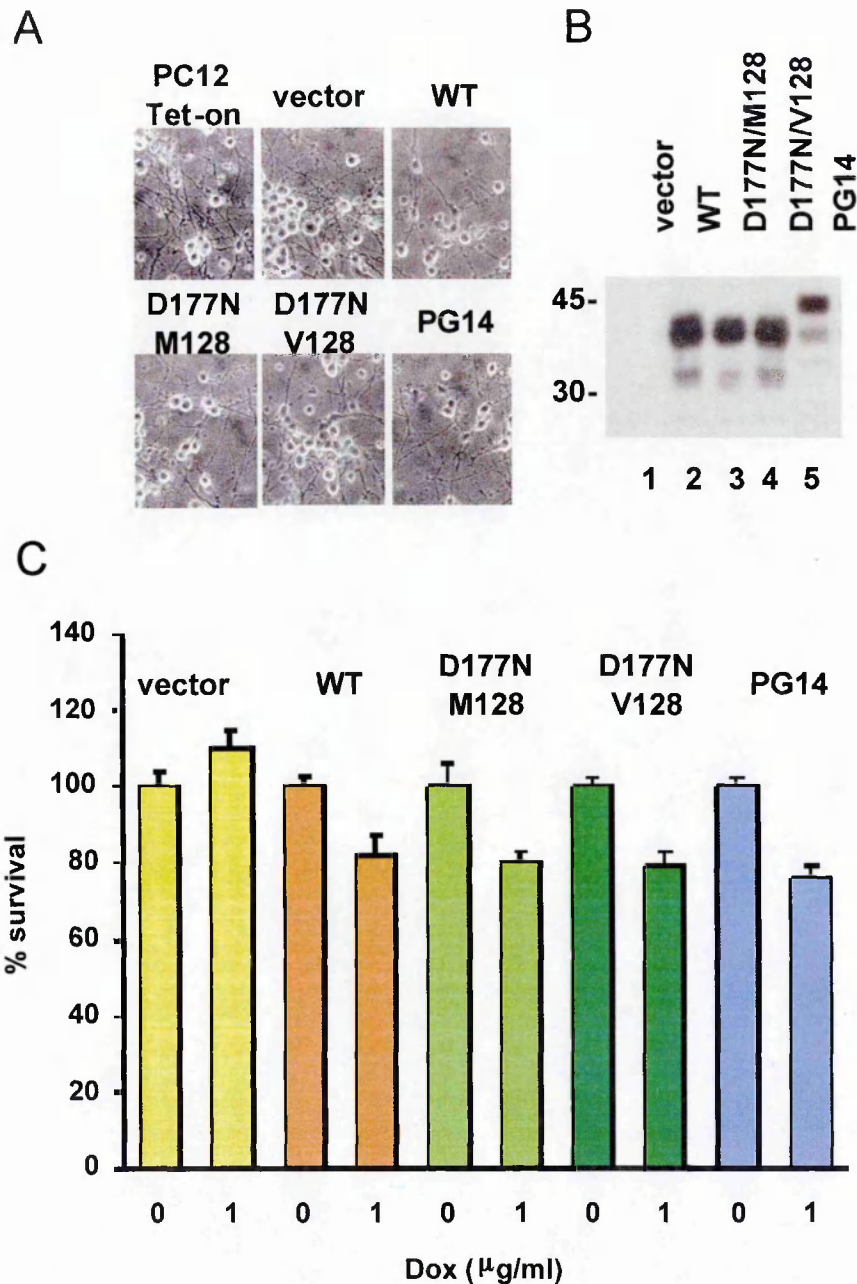


Figure 3.15: Acquisition of neuronal phenotype does not determine a dramatic decrement in cell viability of cells expressing mutant PrP.

(A) Parental PC12 Tet-on cells or cells transfected with the empty vector pBI-G, with WT, D177N/M128, D177N/V128 or PG14 PrP were differentiated as described in chapter 2 and examined by phase contrast microscopy. Pictures were performed on a Nikon F-601M camera.

(B-C) Differentiated PC12 Tet-on cells transfected with the different constructs were seeded on 96-well plates and treated with or without Dox for 96 h.

(B) PrP expression level after Dox induction was checked by lysing one well of cells transfected with the empty vector (lane 1), with WT (2), D177/M128 (3), D177N/V128 (4) or PG14 PrP (5) in laemli buffer followed by Western Blotting.

(C) MTT assay was performed on cells treated with or without Dox. Each value is the mean \pm s.d. of 5-8 wells expressed as a percentage of the survival of control cells.

3.3.2 Expression of mutant PrP does not activate microglial response

Previous studies have demonstrated that addition of microglia to neurons significantly increased the neurotoxicity induced by PrP^{Sc}-like peptides (Bate et al., 2002; Brown et al., 1996; Giese et al., 1998), suggesting that microglia contribute to the elimination of neurones sublethally damaged by PrP^{Sc}. On the basis of these observations we tested whether PC12 cells expressing mutant PrP molecules with scrapie-like properties were recognized as abnormal and killed by microglia. Clones transfected with WT, D177N or PG14 PrPs or with the empty vector, and then differentiated with NGF, were seeded in a 96 wells-plates. After 24 h purified primary rat microglia was added to the PC12 cultures. PC12 cells alone or PC12 cells co-cultured with microglia were treated for 48 h with 0-1 µg/ml Dox before assaying cell viability. No difference in cell survival was found between pure PC12 cell cultures and cells co-cultured with microglia, indicating that the presence of microglia did not alter viability of differentiated PC12 cells transfected with mutant PrPs (Figure 3.16A). Transgenic PrP expression was checked by Western blotting in all the clones (Figure 3.16B).

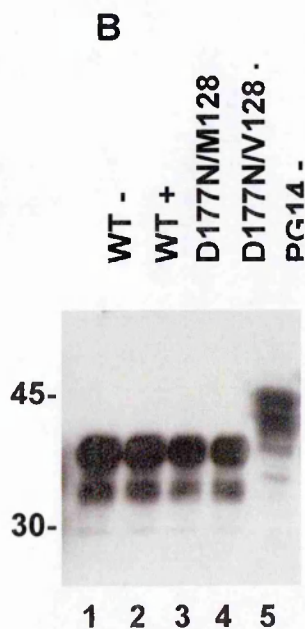
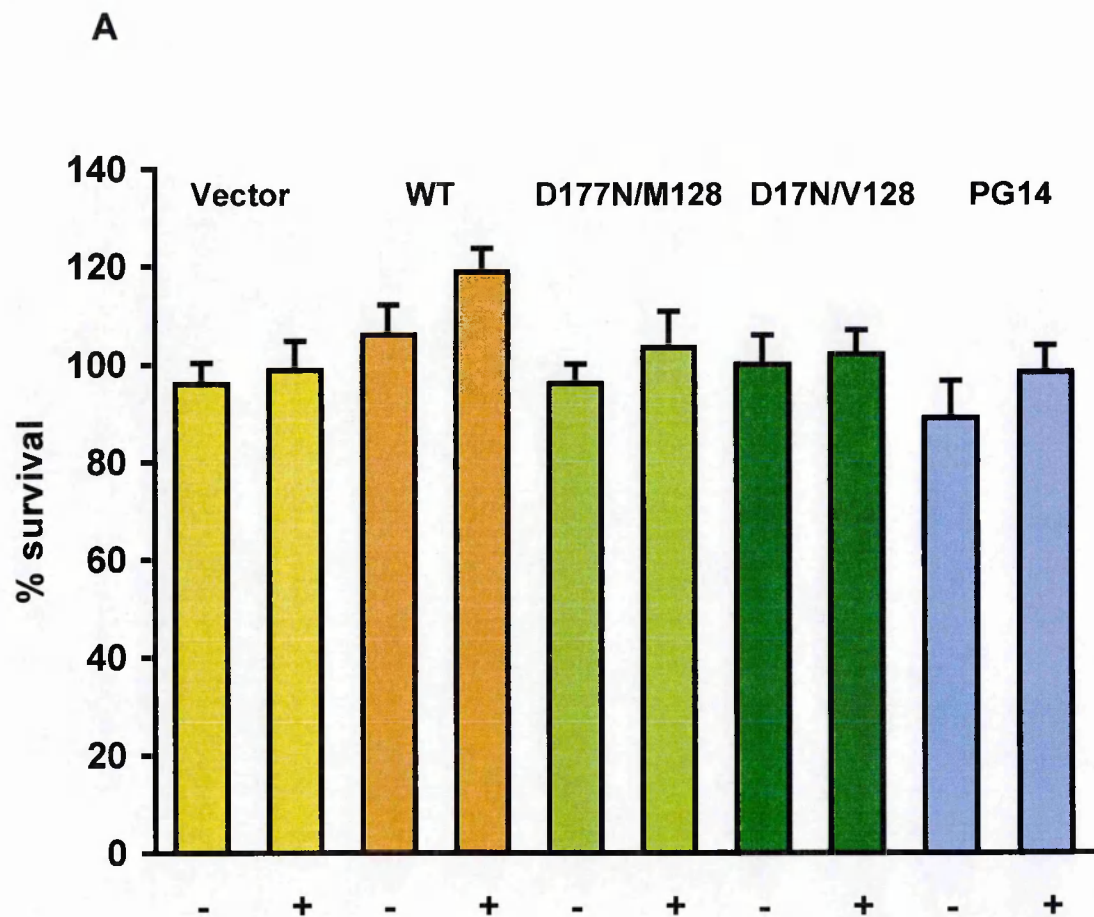


Figure 3.16: Microglia does not kill cells expressing mutant PrP.

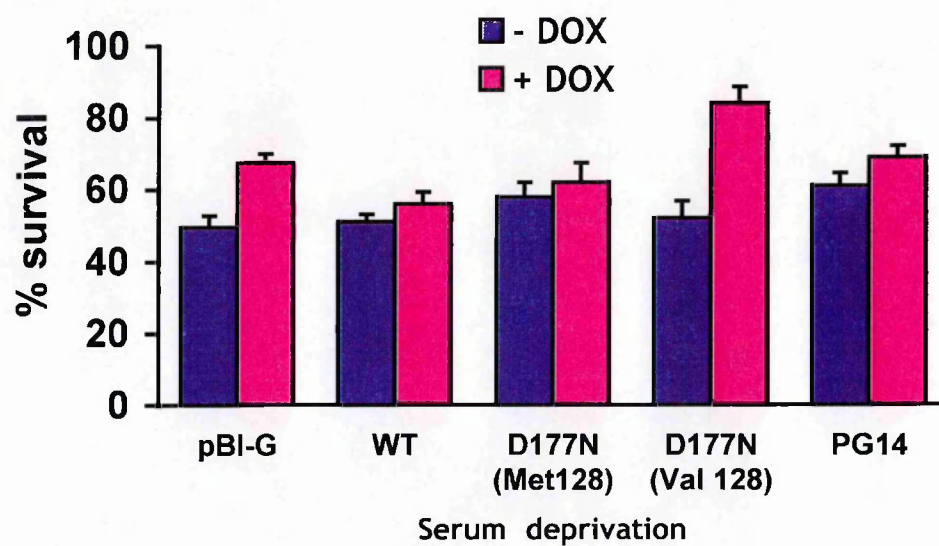
(A) Cells transfected with the empty vector, with pBI-G/WT PrP, pBI-G/D177N/M128 PrP, D177N/V128 or pBI-G/PG14 PrP were differentiated and seeded in a microtiter plate in the presence (+) or absence (-) of microglia. Cells were incubated with 1 μ g/ml Dox for 48 h before performing MTT assay. Each value is the mean \pm s.d. of 5-8 wells expressed as a percentage of the survival of the control cells. (no Dox, with or without microglia).

(B) One well seeded with PC12 cells expressing WT PrP (lane 1 and 2) or expressing D177N/M128 PrP (3), D177N/V128 PrP (4) or PG14 PrP (5) were lysed in laemli sample buffer and moPrP was analysed by Western blotting. PC12 cells analysed in lane 2 were co-cultured in the presence of microglia to check for the influence of microglia on moPrP expression.

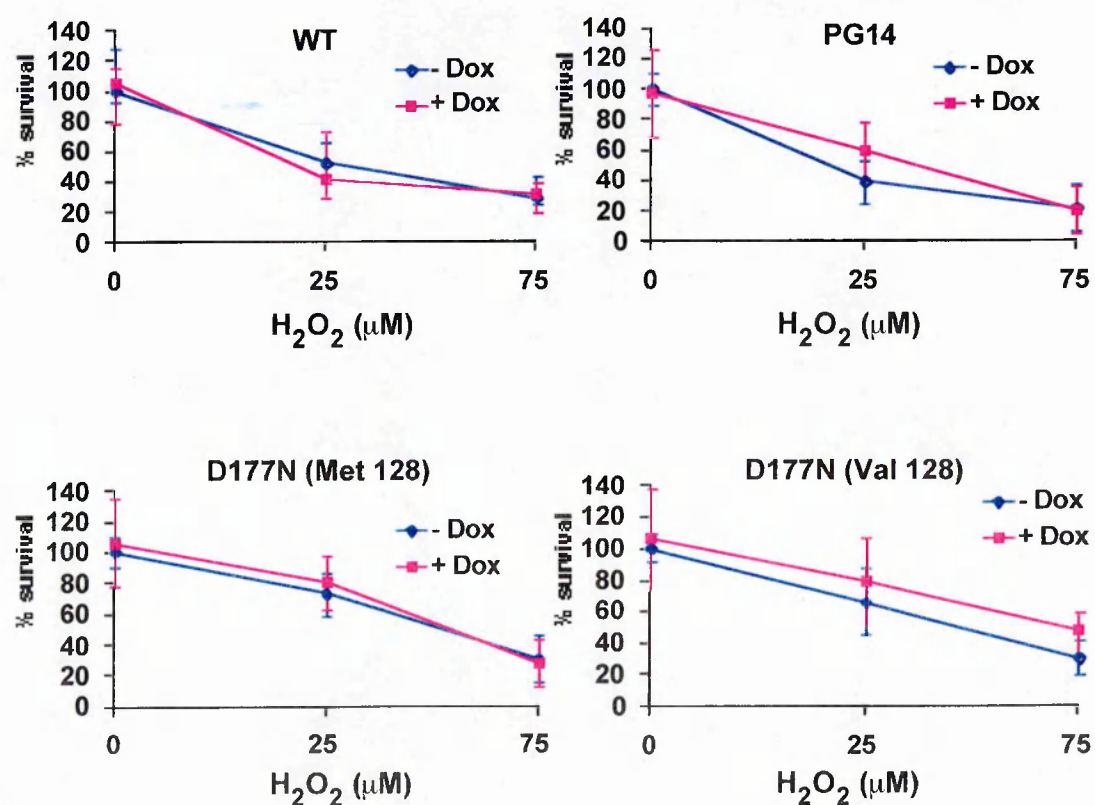
3.3.3 PrP expression does not alter response to cellular stress

It has been reported that PrP can protect neurones from apoptosis induced by serum deprivation (Kim et al., 2004; Kuwahara et al., 1999) and from oxidative stress (Brown et al., 1997b; White et al., 1999). To evaluate the protective role of WT PrP in our cell model and the possibility that mutant PrP could exacerbate the effect of stress inducers, cell viability was evaluated in cells cultured after serum deprivation or in the presence of 0-75 μ M H₂O₂. Cells transfected with the empty vector, with pBI-G/WT PrP, pBI-G/D177N PrP in the two forms or with pBI-G/PG14 PrP were cultured in the absence of serum before and after treatment with Dox. Cell viability was compared to the viability of cells cultured in regular medium (Figure 3.17A). Alternatively, cells transfected with pBI-G/WT PrP, pBI-G/PG14 PrP or with pBI-G/D177N PrP in the two forms were treated with 0-75 μ M H₂O₂ for 24 h before and after induction with Dox (Figure 3.17B). As shown in Figure 3.17 A and B, the viability of cells grown in the presence of Dox did not differ significantly from that of cells grown in the absence of Dox. These results indicated that neither WT or mutant PrP expression affected the cellular response to the two stressors tested. In some cases, there was a slight increase in survival of Dox-treated cells (Figure 3.17A); however this effect was not ascribable to PrP expression, as it was present even in the case of cells transfected with the empty pBI-G vector. As described above, expression level of transgenic PrP was checked by Western blotting (Figure 3.17C and 3.17D).

A



B



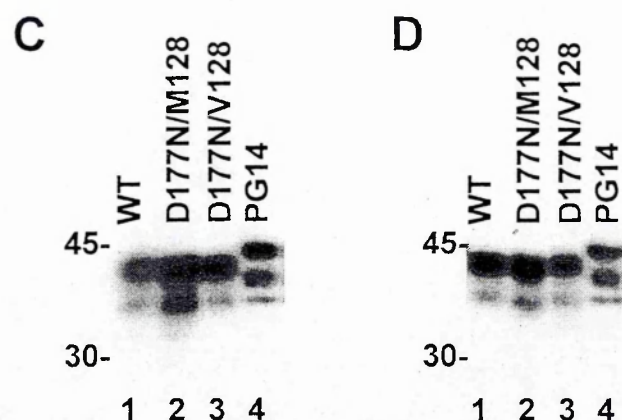


Figure 3.17: PrP expression does not alter viability of cells grown after serum deprivation or in the presence of H₂O₂.

(A) Cells transfected with pBI-G, pBI-G/WT PrP, pBI-G/PG14 PrP, pBI-G/D177N(Met128) PrP or pBI-G/D177N(Val128) PrP were treated with 0 or 1 μ g/ml Dox. After 24 h the regular medium was replaced with serum-free medium and cell viability assessed by MTT after 18 h. Each value is the mean \pm s.d of 5-8 wells expressed as a percentage of the survival of control cells grown in regular medium and in the absence of Dox.

(B) Cells transfected with pBI-G/WT PrP, pBI-G/PG14 PrP, pBI-G/D177N(Met128) PrP or pBI-G/D177N(Val128) PrP were treated with 0, or 1 μ g/ml Dox. After 24 h of Dox induction, cells were treated with 0, 25 or 75 μ M H₂O₂ for 18 h before assaying cell viability by MTT. Each value is the mean \pm s.d of 5-8 wells expressed as a percentage of the survival of control cells (no Dox, no H₂O₂).

(C-D) PrP expression level achieved after Dox induction was checked by Western blotting. Cells transfected with WT PrP (lane 1), D177N/M128 PrP (2), D177N/V128 PrP (3) or PG14 PrP (4) and treated for 24 h with 1 μ g/ml Dox were lysed in laemli sample buffer before loading on SDS gel. Panel C shows PrP expression level of cells before serum deprivation, panel D shows PrP expression level of cells before H₂O₂ treatment.

The drug tunicamycin inhibits protein glycosylation, causing protein accumulation in the ER and activation of ER stress response (Dorner et al., 1990; Leavitt et al., 1977). Based on the assumption that misfolded PrP accumulate in the ER, it was hypothesized that ER stress (induced by tunicamycin) could affect PrP distribution and/or cell viability. Moreover, because PrP is normally glycosylated, this drug acts directly on PrP. Immunofluorescence experiments were performed on cells treated with Dox and tunicamycin. This analysis showed no differences in PrP distribution after tunicamycin treatment (Figure 3.18A), although the drug efficiently inhibited the glycosylation of the protein (Figure 3.18B and 3.19B). The viability of cells in the presence of tunicamycin was then tested. Cells transfected with WT, D177N/M128, D177N/V128 or PG14 PrPs were treated with tunicamycin (2.5 or 5 μ g/ml), with Dox alone, or were co-treated with tunicamycin and Dox. The presence of tunicamycin caused a marked reduction in cell viability in all clones analysed (probably due to the toxicity of the drug itself) which was independent of the presence or absence of Dox (Figure 3.19A). MoPrP expression level was equivalent in all the clones used in this experiment (Figure 3.19B).

Altogether these results indicate that neither deprivation of trophic factors, nor oxidative stress by H_2O_2 or induction of ER-stress by tunicamycin affected the extent of cell death after mutant PrP expression.

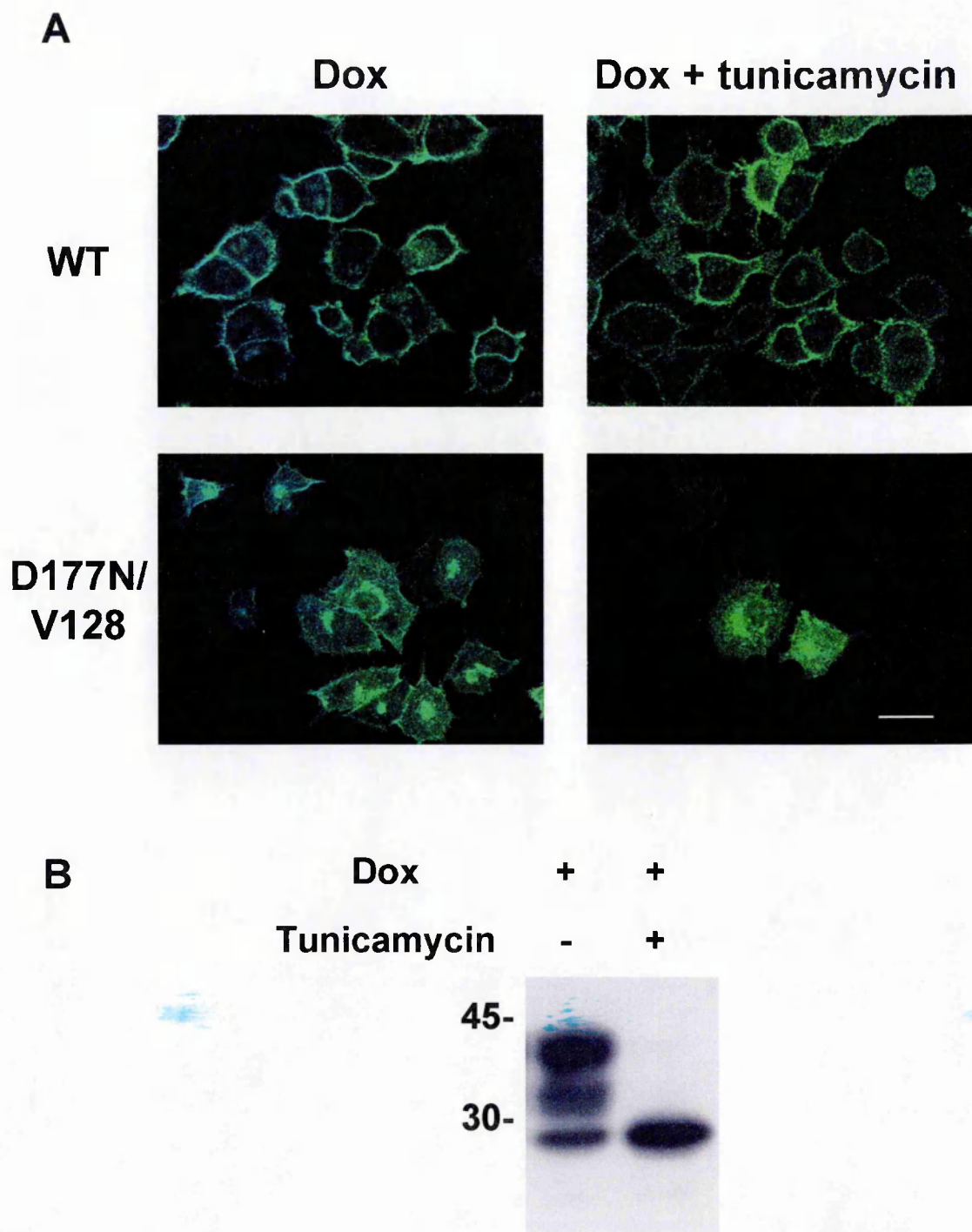


Figure 3.18: Treatment with tunicamycin does not alter the intracellular distribution of PrP.

Cells transfected with pBI-G/WT PrP or pBI-G/D177N/V128 PrP were treated with 1 μ g/ml Dox or co-treated with 1 μ g/ml Dox and 1 μ g/ml tunicamycin for 24 h.

(A) Cells expressing WT PrP and D177N/V128 PrP were fixed, permeabilized and stained with 3F4. Scale Bar is 20 μ m.

(B) Cells expressing WT PrP and treated with or without tunicamycin were lysed in Laemli buffer and analyzed by Western blot using anti-PrP antibody 3F4. After treatment with tunicamycin a single band, running at 27 kDa and corresponding to the unglycosylated form of PrP, was detected.

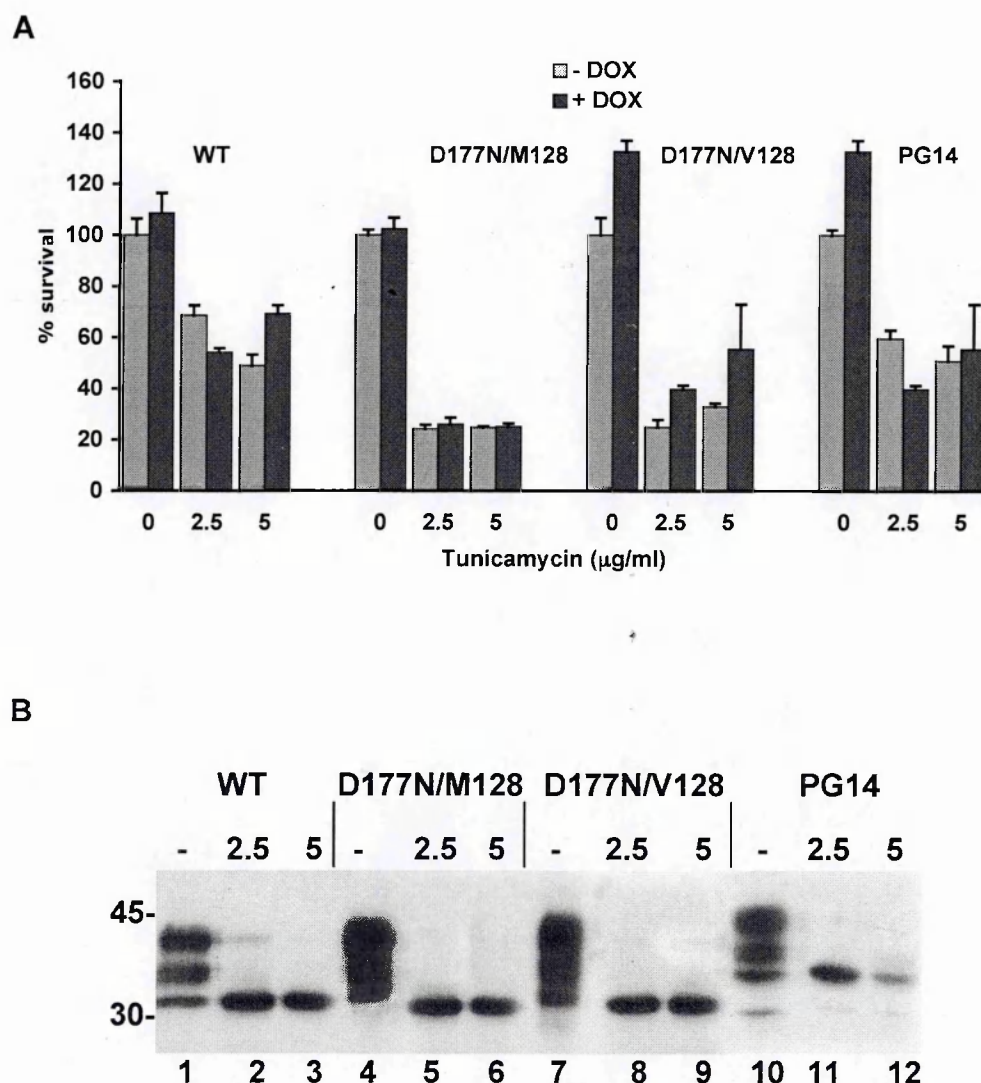


Figure 3.19: WT or mutant PrP expression does not alter susceptibility to tunicamycin.

Cells transfected with WT PrP, D177N/M128, D177N/V128 or PG14 were treated with 0 or 1 µg/ml Dox. After 24 h cells were incubated with 0, 2.5 or 5 µg/ml tunicamycin for 18 h before performing MTT assay (A) or lysis (B).

(A) The bar graph shows the result of MTT assay. Each bar represents the mean \pm s.d. of 5-7 replicates.

(B) After 24 h of induction cells expressing WT (lanes 1-3), D177N/M128 (4-6), D177N/V128 (7-9) or PG14 PrP (10-12) and treated with the indicated concentrations of tunicamycin (0, 2.5 or 5 µg/ml) were analysed by Western blotting.

3.4 Does mutant PrP trigger ER stress?

3.4.1 Analysis of ER stress markers in PC12 Tet-on cells

To explore the possibility that expression of D177N or PG14 PrPs triggered toxic response pathways in the ER, the mRNA level of the molecular chaperon BiP (GRP78) and transcriptional factor CHOP-10 (GADD153) were measured. mRNA levels of BiP and CHOP were analysed in cells transfected with WT, M128V, D177N or PG14 PrP after 24 h of induction with 1 μ g/ml Dox. As shown in Figure 3.20A, PrP expression did not alter the amount of BiP or CHOP transcripts. IRE1a-dependent splicing of XBP1 mRNA, which constitutes one of the first events in ER-stress (Kaufman, 1999; Lee et al., 2002), was then assessed. RT-PCR analysis was performed on mRNA isolated from tunicamycin- or Dox-treated cells (Figure 3.20B). While the spliced form of XBP1 was readily detected after tunicamycin treatment, no changes were detected in cells exposed to Dox in any of the clones analysed. Again, the induction of PrP achieved in these experiments was equivalent, as revealed by Western blot analysis (Figure 3.20C). Because the highest changes in cell viability had been detected after 96 h of mutant PrP induction, the same experiments were performed on mRNA extracted from cells induced with 1 μ g/ml Dox for 96 h. Again, no increase of the ER-stress markers analysed was detected.

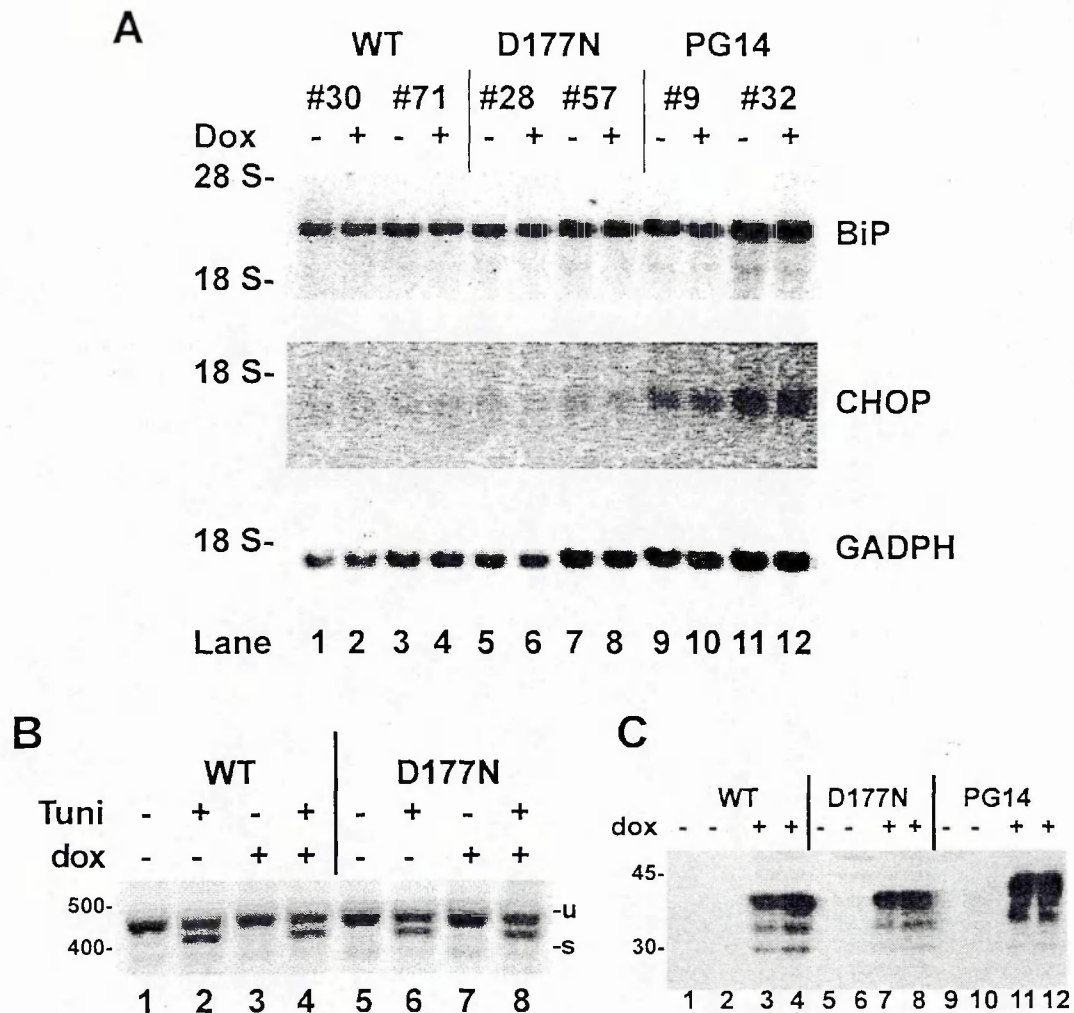


Figure 3.20: Mutant PrP expression does not trigger ER stress response.

(A) Two clones transfected with pBI-G/WT PrP (lanes 1-4), two clones transfected with pBI-G/D177N/M128 PrP (5-8) and two clones transfected with pBI-G/PG14 PrP were induced for 24 h with 0 or 1 μ g/ml Dox. 20 μ g of total RNA extracted were analysed by Northern Blot, using anti-BiP (upper panel) or anti-CHOP (middle panel) specific probes. CHOP and BiP expression level was normalised on the level of expression of GADPH (lower panel). The mutant D177N/V128 did not behave differently from the other constructs and was not included in this figure.

(B) Alternatively, cells expressing WT or D177N/M128 PrP were treated for 24 h with 1 μ g/ml Dox (lanes 3, 4, 7, 8), in the presence (lanes 4, 8) or absence of tunicamycin (lanes 3, 7) or treated with tunicamycin in the absence of Dox (2, 6). Total RNA was extracted and the presence of XBP1 alternative splicing was analyzed by RT PCR. Each product of amplification was separated on 1% agarose gel. Spliced forms (s) were detected only in samples treated with tunicamycin. RT-PCR analysis of clones transfected with PG14 or D177N/V128 PrP gave the same result and was not included in this picture.

(C) Each clone transfected with WT PrP (lanes 1-4), D177N/M128 PrP (5-8) or PG14 PrP (9-12) was analysed for PrP expression before (1-2, 5-6, 9-10) and after Dox induction (3-4, 7-8, 11-12) by Western blotting.

Since only 5% of cells expressing mutant PrPs display an altered intracellular distribution of PrP and only between 2 and 5% of these cells underwent apoptosis, it was possible that only a limited number of cells accumulated sufficient amount of mutant PrP in the ER to activate ER-stress response leading to apoptosis. If this were the case, then analysis of total RNA extracted from whole cultures could contain low, undetectable levels of BiP, CHOP and XBP1 transcripts. To directly test if UPR markers were activated in the cells accumulating PrP, immunofluorescence experiments were performed using anti-PrP and anti-CHOP antibodies. Cells expressing WT or mutant PrP were treated for 48 h with Dox or for 18 h with tunicamycin and subsequently immunostained with the two antibodies. As expected, 5% of cells expressing PG14 PrP displayed an abnormal ER distribution of PrP; however, no cells showed increased levels of CHOP. Cells treated with tunicamycin showed an intense nuclear staining indicative of CHOP induction (Figure 3.21).

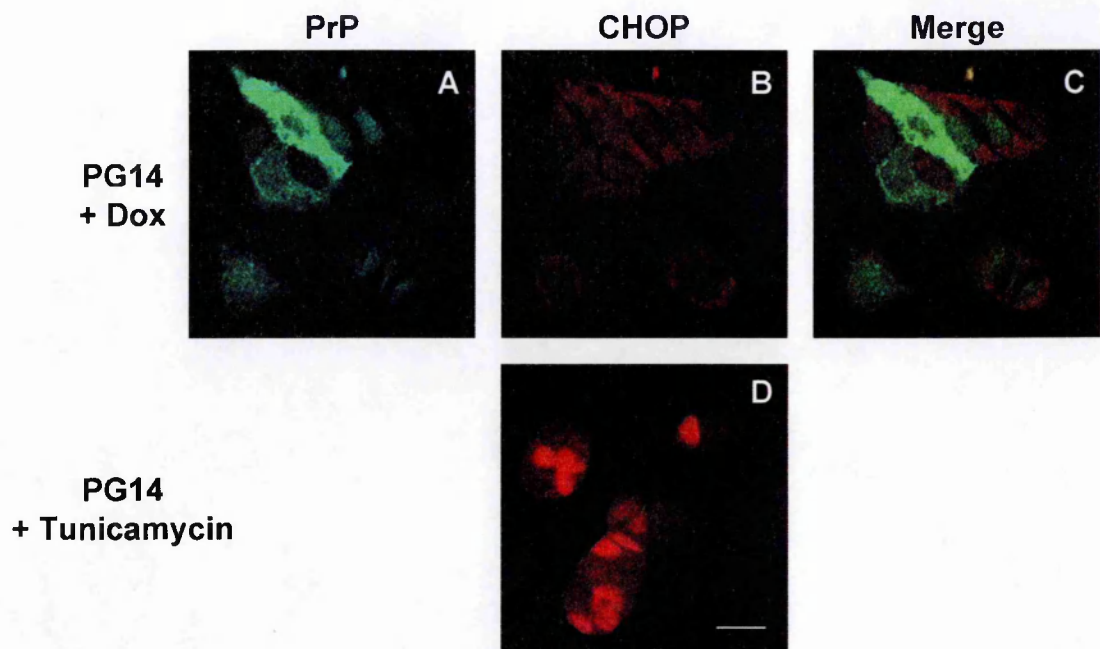


Figure 3.21: CHOP is not activated in cells that accumulate mutant PrP in the ER.

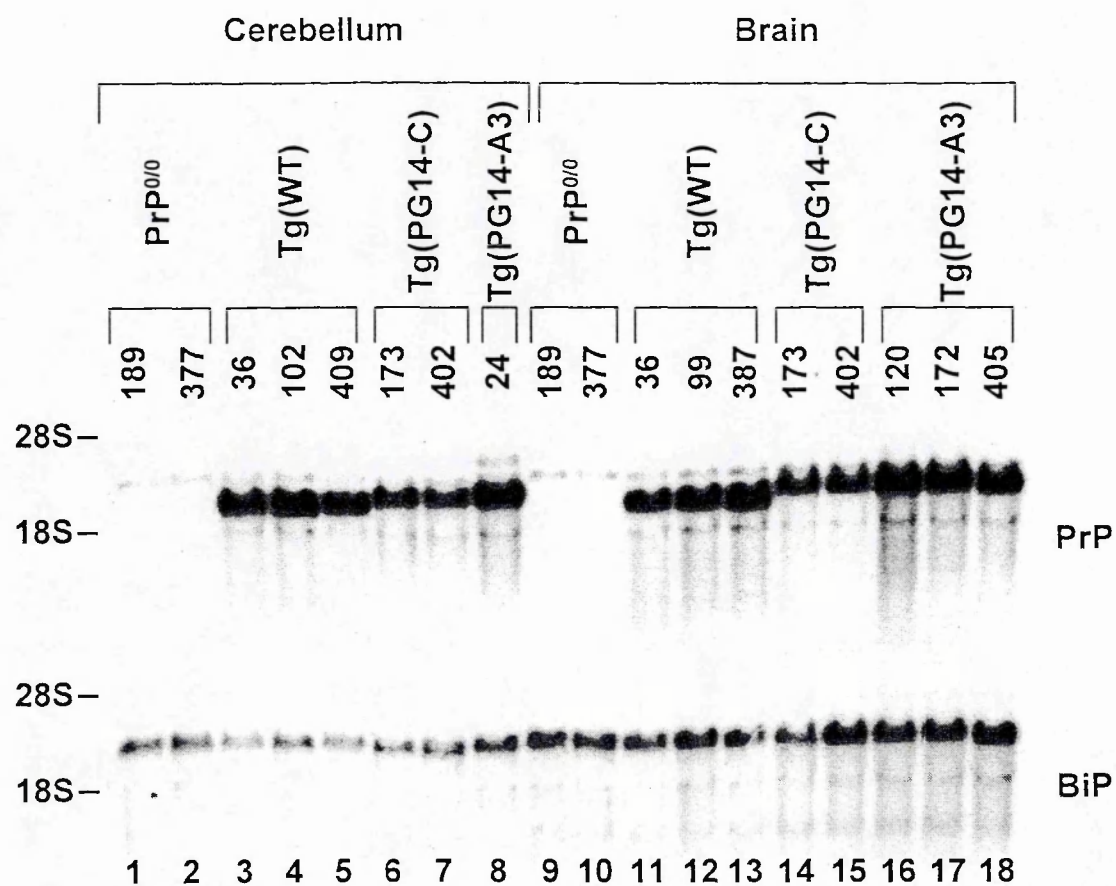
Cells transfected with pBI-G/PG14 were induced for 48 h with 1 $\mu\text{g/ml}$ Dox (panels A, B, C) or for 18 h with 1 $\mu\text{g/ml}$ tunicamycin (panel D). Cells were subsequently fixed, permeabilized and stained with anti-PrP antibody (A) and with anti-CHOP (B) followed by Alexa 488 (green)-conjugated anti-mouse and Alexa 546 (red)-conjugated anti-rabbit. Merged image between the two antibodies is shown in panel C. Cells treated with tunicamycin were stained with anti-CHOP antibody as a positive control for CHOP induction (D). Scale Bar, is 10 μm .

3.4.2 Analysis of ER stress markers in transgenic mice

The activation of the UPR was also analysed in transgenic mice expressing WT (Tg(WT)) or PG14 (Tg(PG14)) PrPs, or mice in which the PrP gene had been ablated (PrP^{0/0}). Accumulation of PG14 PrP in the brains of the Tg(PG14) mice causes a progressive neurological disorder characterized by apoptosis of cerebellar granule neurons (Chiesa et al., 2000; Chiesa et al., 1998). RNA was isolated from the cerebellum or from the whole brain of Tg(PG14) mice at different ages, corresponding to different stages of the disease, as well as from age-matched Tg(WT) and PrP^{0/0} mice. The expression level of BiP mRNA in Tg(PG14) was comparable to that of Tg(WT) and PrP^{0/0} mice (Figure 3.22A) and did not change significantly with aging (Figure 3.22A and 3.22B). CHOP mRNA was not clearly detectable in the CNS of these mice, probably because it is expressed at low level in normal brain (Anguelova et al., 2000; Dimcheff et al., 2003; Reifemberger et al., 1994); however the analysis of the cerebellum of Tg(PG14) mice did not revealed variation in CHOP expression level during aging (Figure 3.22B).

Taken together, these analyses did not reveal evidence for an involvement of the ER stress response as a neurotoxic pathway triggered by mutant PrP accumulation.

A



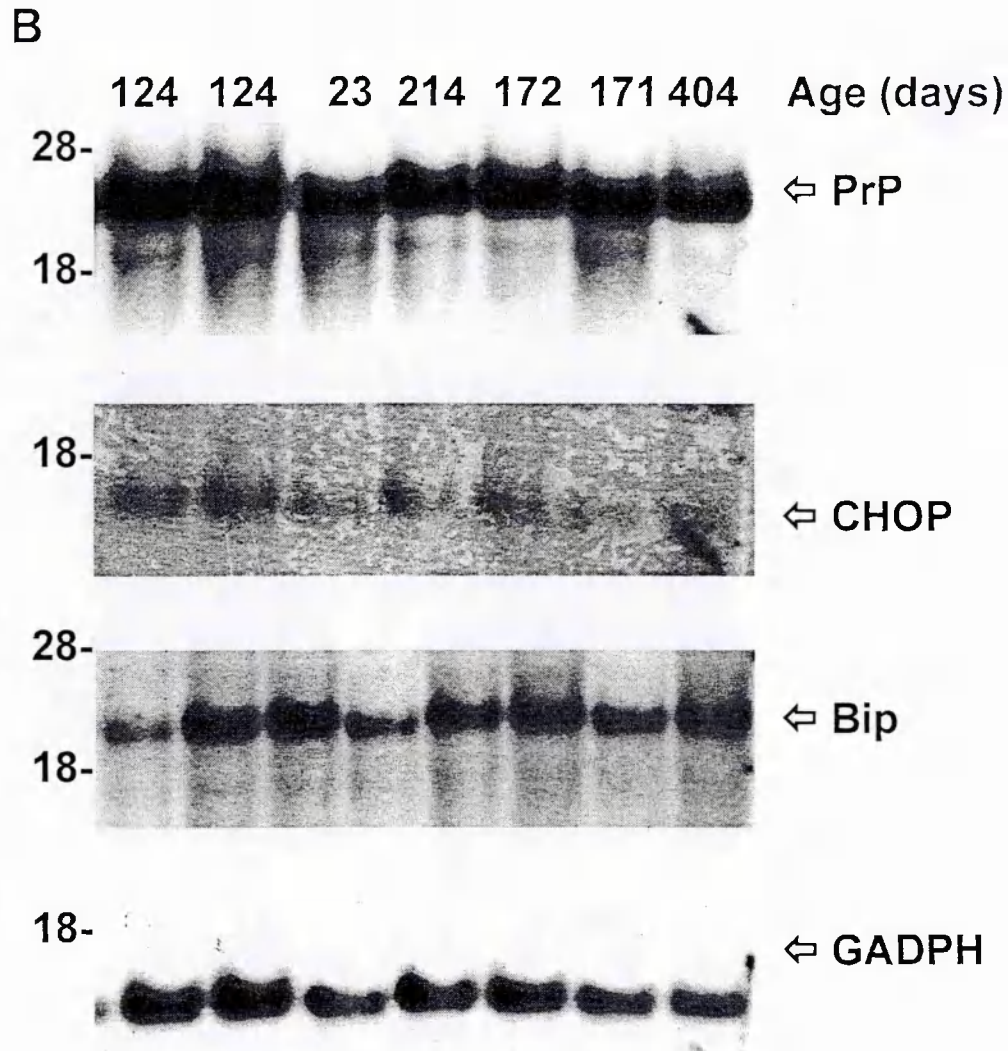


Figure 3.22: Accumulation of mutant PrP in the central nervous system (CNS) of transgenic mice does not induce the expression of BiP or CHOP.

(A) Age-matched PrP^{0/0} mice (lanes 1-2, 9-10), Tg(WT) (expressing 4 times the endogenous level of PrP) (lanes 3-5, 11-13), Tg(PG14-C) (lanes 6-7, 14-15) (expressing 0.3 times the endogenous level of PrP) or Tg(PG14-A3) (expressing 1 time the endogenous level of PrP) (lanes 8, 16-18) were sacrificed at the indicated ages. Total RNA was extracted from the cerebellum (1-8) or from total brain (9-18) and 20 µg were analysed by Northern blot using PrP and BiP-specific probes.

(B) Tg(PG14-A3) were sacrificed at the indicated ages. Total RNA was extracted from the cerebellum and 20 µg were analysed by Northern blot using PrP, CHOP, BiP or GADPH-specific probes.

Chapter 4: Discussion

In this thesis, a cellular model of familial prion diseases has been generated in which PrP expression can be switched on by adding doxycycline. Mutant PrPs expressed acquired PrP^{Sc}-like properties, were retained inside the cell and were sometimes associated with the induction of apoptosis. Since it is known that retention of misfolded protein can lead to stress in the ER and consequently to cell death (Aridor and Balch, 1999), the activation of markers typical of ER stress was investigated. The results presented here suggest that neuronal cell death in familial prion diseases is not linked to ER stress.

4.1 Generation of PC12 Tet-on expressing WT and mutant PrPs

Mouse PrP molecules modified to carry the epitope for the 3F4 antibody (Kascsak et al., 1987) were expressed in PC12 Tet-on cells. The presence of this epitope does not affect the properties of the protein (Scott et al., 1993b) and makes it possible to distinguish transfected PrP from endogenous rat PrP. Moreover, the 3F4 antibody presents several advantages: first, has a very high affinity for PrP; secondly its epitope (aminoacids 109-112) is inside the protease resistant core of the protein, and thus the 3F4 antibody recognizes not only the full length protein but also the proteolytic fragment resulting from digestion of the protein with proteinase-K (Kascsak et al., 1987); and third, the 3F4 epitope is buried in native PrP^{Sc}, thus allowing selective recognition of PrP^{Sc} from PrP^C by immunoprecipitation or by ELISA in non-denaturing conditions (Chiesa et al., 2003; Peretz et al., 1997; Safar et al., 1998).

3F4-tagged mouse PrPs were cloned into the Pst I / Not I restriction sites of pBI-G. The sequencing of the constructs revealed an insertion of 300 bp upstream from the Pst I restriction site of pBI-G/WT PrP. This insertion was probably due to a rare, non-specific event of recombination between molecules of pBI-G/WT PrP or between pBI-G and non ligated insert and was not observed when the experiment was repeated a second time.

The capability of Dox to modulate expression was first checked by assaying β -galactosidase activity after transient transfection of the empty pBI-G alone or in combination with pTet-tTS. In the first case, the comparison between non induced cells and cells induced with 1 μ g/ml Dox demonstrated that the expression was not completely silenced in the absence of the inducer. To avoid leakage, it was decided to introduce the transcriptional silencer pTet-tTS, even if the number of expressing cells and the absolute expression level was reduced. The addition of tTS reduces background expression in mammalian cell lines (Forster et al., 1999; Freundlieb et al., 1999; Rossi et al., 1998) and in transgenic animals (Perez et al., 2002; Zhu et al., 2001) even if sometimes affects the maximal expression level (Knott et al., 2002). The introduction of tTS allowed the selection of clones with no basal activity, but was not possible to achieve high level of expression. However, this expression level, that was comparable to PrP expression in PC12 cells, allowed studies of toxicity in a physiologic context.

4.2 Stable transfection of pBI-G/PrP and analysis of protein expression

In stable transfections, the percentage of cells expressing PrP was clearly lower than the number of cells with β -gal activity after transient transfection. This difference was not due to a different transfection efficiency between the two methods (see table III.II and III.III) but probably reflected the fact that protein expression was analysed at different times after transfection (48 h after transient transfection, more than 4 weeks after stable transfection). In fact, it is known that the expression of a transgene is maximum 24-48 h post-transfection (Colbere-Garapin and Garapin, 1983), and decreases afterwards. During the first 24-48 h, all the cells in which plasmid has entered activate transcription and translation of the gene of interest. However, in the majority of the cases, the plasmid is not integrated into the cellular genome and is lost or degraded. In contrast in the few cells in which the plasmid has been integrated the expression of the gene of interest will persist. To select cells that have been transfected with the plasmid and avoid loss of the plasmid, cells are continuously grown in medium containing the selective agent (Colbere-Garapin and Garapin, 1983).

PrP expression level was well modulated by different doses of the inducer, and increased with increasing concentration of Dox, reaching a maximum at 1 μ g/ml. Northern Blot experiments performed on induced cells, using a probe recognizing both mouse and rat PrPs, revealed that at 1 μ g/ml, the amount of transcript was equivalent to the endogenous rat mRNA level. The distinction between the two mRNA forms was possible because of the

difference in molecular weight. No further increased expression level was detected using higher concentration of the inducer (2-5 $\mu\text{g/ml}$). Moreover, after 72-96 h of induction with 5 $\mu\text{g/ml}$, deleterious effects on cells were clearly observed: this effect was not related to PrP expression but was due to toxicity of Dox itself, as demonstrated by comparing the viability of cells expressing PrP versus cells transfected with the empty pBI-G.

The time course of PrP expression demonstrated that the induction was rapid and lasted at least 72-96 h, while after this period the expression level started to decrease in all clones analyzed. The explanation for this finding is not clear, although this phenomenon in PC12 Tet-on cells expressing Bcl-2 has been previously described (Schwarz et al., 2002).

In previous studies mouse PrP was expressed in N2a cells using either the Tet-on or the Tet-off system (Windl et al., 1999). In that model, treatment with Dox revealed a leakage of inducibility in N2a Tet-off, while a good expression level was achieved in N2a Tet-on cells. The quantitative regulation of PrP expression in N2a Tet-on cells was examined by inducing expression for 48 h with various concentration of Dox. PrP expression was detected at 5 ng/ml and the signal reached saturation at Dox concentrations around 500/1000 ng/ml. The maximum expression level achieved was much higher than that in normal mouse brain and that in cells expressing PrP under the control of a constitutive viral promoter. The time-course revealed induction of PrP after 3-5 h, with a maximal expression level after 20 h. The effect of longer treatments was not shown. Wild-type PrP was also expressed in RK13 Tet-on (Rachidi et al., 2003). PrP induction was Dox-dependent, being detectable at 10 ng/ml and reaching a maximum at 500 ng/ml of Dox. Kinetics of induction with 500 ng/ml showed that expression was detected after 8 h, reaching a maximum after 24h.

Again the effect of longer treatments was not shown. Therefore, in N2a Tet-on (Windl et al., 1999), in RK13 Tet-on (Rachidi et al., 2003) and in PC12 Tet-on, PrP has a similar dose-dependence and kinetic of induction.

Finally, in the system developed in this thesis it was possible to activate and inactivate transcription by repeatedly adding and removing Dox, thereby demonstrating that the inducibility of the system was not lost after the first cycle of induction.

These pilot experiments therefore demonstrated that the system developed did operate and allowed determination of the best experimental conditions for use in subsequent studies.

4.3 Mutant PrPs expressed in PC12 Tet-on acquire abnormal biochemical properties and are less represented on the cell surface compared to WT PrP

Several cellular models of familial prion diseases have been previously established in which mutant PrPs were constitutively expressed from viral promoters. In those systems, mutant PrP acquired abnormal properties reminiscent of PrP^{Sc}, such as detergent insolubility and PK resistance (Chiesa and Harris, 2000; Daude et al., 1997; Lehmann and Harris, 1996a; Lehmann and Harris, 1996b; Negro et al., 2001; Priola and Chesebro, 1998). In this thesis, an inducible model was developed in which expression of transfected PrP was not constitutive but was induced by doxycycline added to the culture medium. For all the different PrPs expressed (WT or mutant), the most abundant form was the di-glycosylated form, with the un-glycosylated least present. This result demonstrate that transgenic PrP is recognized by the

cellular machinery and suggests that the presence of a mutation in the PrP molecule does not intrinsically alter the ratio among the glycoforms. Previous studies have shown that the presence of the mutation at codon 178 of human PrP (or 177 of murine PrP) increase (Lehmann and Harris, 1996a) or decrease (Petersen et al., 1996) the amount of the un-glycosylated form in respect of WT PrP. These apparent discrepancies probably reflect differences in cell lines used and in PrP primary sequence: in fact, while Lehmann et al. (1996) used mouse PrP (without the 3F4 tag) transfected into CHO cells, Petersen et al. (1996) used human PrP transfected into M-17 human neuroblastomal cells.

Consistent with the results obtained in constitutive expression models (Chiesa and Harris, 2000; Daude et al., 1997; Lehmann and Harris, 1996a; Lehmann and Harris, 1996b; Negro et al., 2001; Priola and Chesebro, 1998) the mutant PrPs, carrying either the D177N mutation in the two forms (linked to M128 or M128V) or the 9 octapeptide insertion (PG14) in the present (inducible) system became detergent insoluble and all developed resistance to protease. The glycoform ratios of insoluble and PK-resistant PrP were similar, suggesting that the three glycoforms have the same propensity to misfold.

The ability of mutant PrP to acquire abnormal biochemical properties in a Tet-on model has recently also been demonstrated in murine neuroblastoma (N2a) cells expressing a murine PrP in which all the alanine residues of the sequence AGAAAAGA (spanning aminoacids 109-112) were replaced by glycine residues (Wegner et al., 2002). This "artificial" mutant protein became resistant to PK digestion 20 h after synthesis. The data presented in this thesis demonstrate that pathogenic mutants linked to familial prion diseases also acquire abnormal biochemical properties when expressed using a Tet-on system.

In PC12 Tet-on cells (as well as in N2a Tet-on) acquisition of PrP^{Sc}-like properties is not impaired by long-term treatment with doxycycline, a drug that has been reported to have anti scrapie properties (Forloni et al., 2002). In fact it was shown that incubation with doxycycline at concentration ranging from 10 μ M to 1 mM resulted in a dose dependent decrease in protease resistance of PrP^{Sc} purified from infected hamster brains. These concentrations, effective in those studies, however, are 1000 fold higher than concentration of Dox used to activate transcription in the Tet-on system. Moreover, before transfecting PC12 Tet-on cells, the effect of doxycycline on the PrP^{Sc} properties of mutant PrP expressed in stable transfected PC12 cells was tested. No differences in detergent insolubility or PK resistance of mutant PrP was found between cells treated with Dox compared to non treated cells at the levels of Dox used in the Tet-on system.

Several studies have demonstrated that the presence of a mutation in the PrP sequence intrinsically favours the conformational change towards a β -sheet-rich state. First, recombinant PrP carrying pathogenic mutations aggregated into inclusion bodies when expressed in *Escherichia coli*. (Liemann and Glockshuber, 1999; Swietnicki et al., 1998). Second, synthetic peptides spanning human PrP residues 169-185 carrying the D178N showed increased aggregation compared to WT sequence (Forloni et al., 1999). Third, mutant PrP expressed in cultured cells acquired abnormal biochemical properties reminiscent of PrP^{Sc} (Chiesa and Harris, 2000; Daude et al., 1997; Lehmann and Harris, 1996a; Lehmann and Harris, 1996b; Negro et al., 2001; Priola and Chesebro, 1998).

Pulse chase experiments performed on transfected cells expressing mutant PrPs demonstrated that PrP^{Sc}-like properties were acquired in a

stepwise fashion by new synthesized PrP molecules (Daude et al., 1997; Lehmann and Harris, 1996b). The earliest biochemical change that was detected within minutes of pulse-labelling cells was resistance to PIPLC. This observation suggested that an initial alteration in the mutant PrP (that probably reflected an intrinsic hydrophobicity of the molecules) occurred in the ER or very soon after translation of the polypeptide chain. The second change was acquisition of detergent insolubility, which was not maximal until 1 h of chase, occurred in a post-Golgi location and presumably reflected aggregation of PrP. Finally, mutant PrP acquired protease resistance, several hours after labelling, demonstrating that acquisition of detergent insolubility and PK resistance were temporally distinct states. These results argued that the generation of PrP^{Sc} involves intrinsic structural features of the mutant PrP molecule itself and that the efficiency of the process is promoted by organelle-specific factors. The data presented in this thesis suggest that the presence of the mutation confers to the new synthesized protein the propensity to misfold and are therefore in agreement with the pulse-chase experiments carried out in constitutive models (Daude et al., 1997; Lehmann and Harris, 1996b). In particular it was noted that the amount of detergent insoluble PrP increased progressively after induction, reaching a maximum after 48-72 h of Dox treatment.

Quantification of mutant PrP insolubility revealed a greater propensity of PG14 molecules to distribute in the pellet fraction compared to D177N mutations (D177N/M128: 45±7.5%; D177N/V128: 46±9%; PG14: 60±14%; n=3). These data are in agreement with previous analysis of the effect of different mutations (either point substitution or insertional) on detergent insolubility of PrP expressed (Ivanova et al., 2001; Lehmann and Harris, 1996b). In these studies the insertional mutation PG14 was more efficient in

inducing insolubility compared with the point mutations P101L, D177N or E199K. Moreover, previous work demonstrated that in cells transfected with PrP carrying different insertional mutation, the longer the insertion, the more PrP was found in the pellet fraction (Priola and Chesebro, 1998). These data suggest that alterations in the octapeptide region, which lies within the unstructured N-terminus of PrP, confer a greater propensity to form insoluble aggregates than point mutations in the C-terminal region.

The percentage of insoluble PrP was similar for the D177N/M128 and for the D177N/V128, suggesting that the polymorphism at codon 128 did not influence the conformation determined by the mutation D>N at codon 177. According with these data, studies on the thermodynamic stabilities of human PrP(121-231) showed that the exchange of Asp178 by Asn destabilises the variants D178N/M129 and D178N/V129 by a similar value (Liemann and Glockshuber, 1999).

Also, in this thesis, immunofluorescence experiments showed that while WT PrP was expressed on the cell surface, mutant proteins were less represented, with the PG14 construct showing the least extent of surface staining. This phenomenon was not caused by different affinities of the different PrP molecules to antibodies but reflected a different subcellular distribution of the protein. Indeed, PG14 PrP and D177N PrPs were predominantly detected inside the cell, and colocalized with Golgi markers in the majority of cases; in 5% of the cells mutant PrP showed a widespread distribution and colocalized with ER markers, suggesting retention in the endoplasmic reticulum. The reason for this difference in the intracellular distribution among different individual cells from the same clone is unclear but could be due to different expression levels of mutant PrP or to differences in the capability of the cell to

metabolise mutant PrP. In either case, the excess of PrP could accumulate in the endoplasmic reticulum, with consequent damage to the cell. Similar results have also been obtained in other cell lines (Ivanova et al., 2001; Priola and Chesebro, 1998) and in cultures of primary neurons obtained from Tg mice expressing PG14 or D177N PrPs. (Fioriti et al., 2005). In the present studies such PrP was retained in the ER and there was no evidence for the presence of mutant PrP in the cytosol, as was described by Ma and Lindquist (Ma and Lindquist, 2001) in their studies of PrP carrying the D177N mutation transfected in COS cells.

Recently it has been shown that the different subcellular localisation of mutant PrPs is due to a retarded transit towards the cell surface, when compared with WT PrP, rather than delivery impairment (Driscaldi et al., 2003). On the basis of these results it was assumed that the presence of the mutation favoured the misfolding of the protein and that this slowed its delivery to cell surface. One could therefore hypothesise that the propensity of each mutant protein to aggregate would influence the kinetics of its transit towards the cell surface, and thus that mutations that confer to PrP a great propensity to aggregate (like PG14) would cause a marked reduction of cell surface distribution of the protein. The finding that PG14 PrP was more detergent insoluble than the other mutant proteins studied here and showed greater retention in the ER than D177N PrP corroborates this hypothesis.

It has been previously demonstrated that treatment with tunicamycin did not impair transport of WT PrP to the cell surface (Lehmann and Harris, 1997; Petersen et al., 1996) but favoured degradation of unglycosylated PrP carrying the D178N mutation (Petersen et al., 1996). In the PC12 Tet-on model developed in this thesis tunicamycin treatment did not influence subcellular

localisation either of WT PrP, in agreement with previous data, or that of mutant PrPs. These data suggest that PrP biosynthetic trafficking is not altered by the absence of sugar residues, or following ER stress induced by tunicamycin.

Taken together the data described suggest that the presence of the mutation favours the misfolding of PrP expressed in PC12 Tet-on cells. The conformation change favours the retention in the endoplasmic reticulum and induces the aggregated state of the protein but does not alter its post-translational modifications.

4.4 Effects of mutant PrP expression

4.4.1 Prolonged expression of mutant PrP causes the appearance of apoptotic cells

The inducible model allowed us to test if mutant PrP expression could induce cytotoxicity and, conversely, if WT PrP expression could “rescue” cells from death, induced by various stimuli, as described previously (Brown et al., 1997b; Kuwahara et al., 1999; White et al., 1999). The toxicity of PrP^{Sc} has been recapitulated in vitro, by applying PrP^{Sc} purified from infected brains to neurons in culture (Giese et al., 1998; Muller et al., 1993; Post et al., 2000). In particular, more recently, it has been demonstrated that neurons expressing ovine PrP exposed to infected sheep brain underwent apoptosis (Cronier et al., 2004). An alternative strategy has been to analyse the effect on cultured neurons of synthetic peptides derived from the PrP sequence. The peptide spanning residues 106-126 has been found to form protease-resistant, β -sheets-rich aggregates reminiscent of PrP^{Sc} (De Gioia et al., 1994; Jobling et al., 1999; Selvaggini et al., 1993) and caused apoptotic death of cultured

neurons (Brown et al., 1994; Brown et al., 1996; Fioriti et al., 2004; Forloni et al., 1993; Forloni et al., 1994). Conversely, a clear cytotoxic effect of PrP carrying pathogenic mutations had not been previously demonstrated in transfected cells (Chiesa and Harris, 2000; Daude et al., 1997; Lehmann and Harris, 1996a; Lehmann and Harris, 1996b; Negro et al., 2001; Priola and Chesebro, 1998). However, because the expression of mutant PrP was not inducible but constitutive in all those models, it was possible that clones resistant to mutant PrP toxicity had survived after transfection and clonal selection (i.e. the use of selecting agents could have masked the potential toxicity of mutant PrP). The advantage of the model used in the present studies over constitutive models related to the possibility of switching on protein expression only at the time of the experiment, thus avoiding any unwanted selection of clones intrinsically resistant to PrP toxicity. Cells were kept in a Dox-free medium until the time of the experiment; moreover, because doxycycline is often administrated to animals, only bovine and horse sera tested for the absence of this antibiotic were used in this study. The results obtained suggested that the prolonged expression of mutant PrP is associated with a decrease in cell viability and with the appearance of apoptotic cells. In this context it was of interest to note that PG14 PrP has been shown to be a potent trigger of neuronal death *in vivo*, as transgenic mice expressing this protein develop a neurodegenerative illness characterized by apoptosis of cerebellar granule neurons (Chiesa et al., 2000; Chiesa et al., 1998).

The observed phenomenon of increased neural apoptosis in the present study was specific to the mutant PrPs, as it was not detected in cells transfected with WT PrP or with the empty pBI-G, but was not easily reproducible. The explanation of this inconsistency could relate to the variability in the induction

level observed after Dox treatment. In an attempt to correlate PrP expression or its subcellular distribution with apoptosis, TUNEL staining or staining with propidium iodide was performed on cells immunostained for PrP. However, cells with an ER-distribution or showing an high expression level of PrP were not those undergoing apoptosis. Conversely, no PrP signal was detectable in apoptotic cells, probably due to the reduced protein synthesis in cells undergoing apoptosis (Sheikh and Fornace, 1999). However it cannot be excluded that although cells displaying high level of intracellular PrP were not undergoing apoptosis at the time of staining they might not do so as a later event.

It is worth to consider that endogenous, wild-type PrP expression, that is quite similar to the maximum expression level of the transfected PrP, could account for the inconstancy of the toxicity. Even if the mutation in one single allele is sufficient for the development of genetic forms, one could speculate that in this model D177N and PG14 can not act as dominant mutations, due to the species barrier between mouse and rat PrP.

Brain and spinal cord are the sites of primary pathology in prion diseases, raising the hypothesis that neurons are more sensitive to PrP^{Sc} accumulation than other cell types. To test the potential cytopathic effect of mutant PrP expression on neuronal phenotype, PC12 cells were differentiated with NGF before inducing PrP expression. However, it was not possible to detect differences in terms of cell viability between undifferentiated and differentiated cells, demonstrating that PC12 cells that acquire a neuronal phenotype are not more susceptible to mutant PrP expression. In agreement with this finding, previous data showed that in stably transfected PC12 cells, neither the biochemical properties of WT and PG14 PrP nor cell viability

changed after NGF differentiation (Chiesa and Harris, 2000). In this thesis the potential increment of endogenous PrP expression due to NGF treatment (as previously reported by two different groups (Mobley et al., 1988; Wion et al, 1988)) was not investigated. However, the increased expression level of endogenous, wild-type PrP could have masked the toxicity of mutant PrP expressed, that did not vary after differentiation.

Microglial activation proceeds the detection of apoptotic neuronal cell death in scrapie-infected hamster brains (Giese et al., 1998). Moreover, it has been reported that microglia increased cell death induced by PrP^{Sc} purified from infected brain and by neurotoxic PrP peptides in cultured neuronal cells (Bate et al., 2002; Brown et al., 1996; Giese et al., 1998). These data suggest that microglial activation is involved in the neurotoxicity of PrP^{Sc} *in vitro* and *in vivo*. To investigate if microglia could influence cell viability of PC12 Tet-on cells expressing mutant PrPs, we co-cultured differentiated PC12 Tet-on cells with rat microglia. However, our results demonstrated that the addition of rat microglia did not result in increased susceptibility of neurons to the toxic effects of mutant PrP expression. Possible explanations for this negative result are that unlike other systems, microglia is not able to trigger death signalling in neuronal PC12 cells. Second, microglia does not trigger death signalling because it does not express mutant PrP. Finally, change induced on neurones by the mutant PrP are not efficient to activate the microglia.

To test the effect of various toxic stimuli on cells expressing mutant PrP compared to cells expressing WT PrP, different clones were treated with H₂O₂, tunicamycin or were grown in the absence of serum before and after PrP induction. Cell viability assays of the same clone exposed to these stimuli in the induced or non-induced state revealed that expression of mutant PrP did not

render cells more susceptible to the insults. Conversely, in some cases, a mild, non statistically significant increase in the number of cells was noticed after Dox treatment. This increase was not due to a protective effect of the PrP construct, being present even in the absence of the insult and in cells transfected with the empty vector. One explanation of this finding could be due to a slight trophic effect of Dox. Doxycycline has been shown to stimulate proliferation of T-cells (Batinac et al., 2003) and of periodontal ligament cells (Zhao et al., 2003). Moreover, it has been shown that doxycycline protects neurons against apoptosis induced by ionising radiation (Tikka et al., 2001). Finally, these experiments revealed that the over-expression of WT PrP did not rescue cells from death induced by H₂O₂, tunicamycin or serum deprivation. This last finding does not exclude the possibility that WT PrP has a neuroprotective role (as previously demonstrated (Brown et al., 1997b; Kuwahara et al., 1999; McLennan et al., 2004; White et al., 1999) but suggests that additional expression of WT protein to PC12 cells, which normally express endogenous PrP, does not make differences in protecting from toxic stimuli. Finally, the expression of endogenous, wild-type PrP, could explain the lack of increased susceptibility of cells expressing mutant PrP expression towards the toxic stimuli.

In summary, data presented in this thesis show that mutant PrP expression was rarely associated to decrease in the cellular viability. More consistent results could be achieved neither by differentiating cells with NGF nor by co-culturing them with microglia. Finally, mutant PrP expression did not increase susceptibility to various toxic stimuli. To explain the inconstancy of these data it can be hypothesized that toxicity was related to the absolute expression of mutant PrP achieved in each single experiment or to an

undefined experimental condition which favoured death of cell expressing mutant PrPs.

Finally it is possible to speculate that the primary and consistent consequence of mutant prion expression is an alteration in cell signalling that can or cannot lead to cell death (see below).

It is worth to consider that endogenous PrP expression could have interfered in the evaluation of the toxicity triggered by mutant PrP expression. In order to rule out this hypothesis, evaluation of the toxicity of mutant PrP could be performed in cells in which the endogenous PrP has been shut down.

4.4.2 Expression of mutant PrP does not trigger ER stress

Secretory and membrane proteins that are retained in the ER may be subjected to a quality control process in which they are retrotranslocated into the cytoplasm and degraded by the proteasome (Bonifacino and Weissman, 1998; Tsai et al., 2002). This mechanism is meant to ensure that abnormally folded proteins, do not reach the plasma membrane. Two PrP mutants, Y145stop and Q160stop appear to be degraded primarily by the retrotranslocation/proteasome pathway (Lorenz et al., 2002; Zanusso et al., 1999). Conversely, it has been shown that although PG14 and D177N PrPs are delayed in their exit from the ER, they are not subjected to retrograde transport into the cytoplasm to be degraded by the proteasome (Drisaldi et al., 2003). Blockage of proteasome activity in PC12 Tet-on cells did not cause accumulation of PrP, confirming that PG14 and D177N are not degraded by the proteasome in this system. In some other diseases, such as hereditary encephalopathy (PiZ variant), the retained protein accumulates in the ER and

triggers ER stress response pathways without being retrotranslocated into the cytosol (Aridor and Balch, 1999). Up-regulation of ER-stress chaperone proteins have been documented in N2a challenged with PrP^{Sc} and in brains of humans affected with sporadic and variant CJD (Hetz et al., 2003). To test if retention of PG14 and D177N PrP could stimulate ER stress, the expression of markers typical of ER stress was examined. The data demonstrated that neither early UPR markers, such as a XBP1 splicing event, nor BiP or CHOP are induced following expression of PG14 or D177N PrPs. The absence of induction of UPR markers was confirmed *in vivo*, through the analysis of transgenic mice expressing PG14 PrP. The possibility that UPR was a rare event, involving only a few cells, was explored by performing CHOP immunostaining on induced PC12 Tet-on cells, and on cultured neurons from Tg mice. These experiments showed that CHOP was not activated in cells expressing mutant PrPs. Taken together, these data suggest that accumulation of PG14 or D177N PrPs does not trigger ER stress and consequently that ER stress is not the cause of cell death seen in the cell models studied here nor in the brains of Tg(PG14) mice.

D177N or PG14 mutations have not been shown to clearly favour formation of PrP forms partially localized in the cytosol, as ^{Ctm}PrP (Stewart and Harris, 2001), or totally cytosolic (Drisaldi et al., 2003)(Fioriti et al., 2005). Moreover, toxicity of ^{Ctm}PrP (Hegde et al., 1999 ; Stewart and Harris, 2003) and cytosolic PrP (Drisaldi et al., 2003; Ma et al., 2002; Roucou et al., 2003; Wang et al., 2004) is still controversial. For these reasons the presence of cytosolic forms of PrP and the relevance in the pathogenicity of the D177N and PG14 mutations were not investigated accurately in this thesis.

Alternatively it is possible to speculate that mutant PrP molecules that are responsible for neurodegeneration are not the ones that are retained

intracellularly, but those that reach the cell surface, by altering the normal physiologic role or by a gain of function of the protein. In this case PrP might damage cells by altering membrane properties, or interacting abnormally with other proteins (Figure 4.1). Recently Tg(PG14) mice were crossed to Bax^{-/-} mice, in which the pro-apoptotic gene Bax had been ablated to obtain Tg(PG14)/Bax^{-/-} offspring (Chiesa et al., 2004). Clinical and neuropathological evaluation of these double Tg mice revealed that deleting Bax was enough to rescue cerebellar granule cells from apoptosis but did not affect the development of clinical symptoms nor synaptic loss and shrinkage in the molecular layer of the cerebellum. These results indicate that synaptic abnormalities induced by accumulation of PG14 PrP contribute significantly to the neurological syndrome seen in Tg(PG14) mice and therefore support the hypothesis that PrP present on cell surface is indeed responsible for occurrence of the disease. Injury to synapses may lead secondarily to death of granule neuron, via Bax activation. Synaptic damage has been documented in models of murine scrapie (Jeffrey et al., 2000a) and in sporadic and inherited CJD (Jeffrey et al., 2001; Kitamoto et al., 1992), suggesting a common role in prion disease.

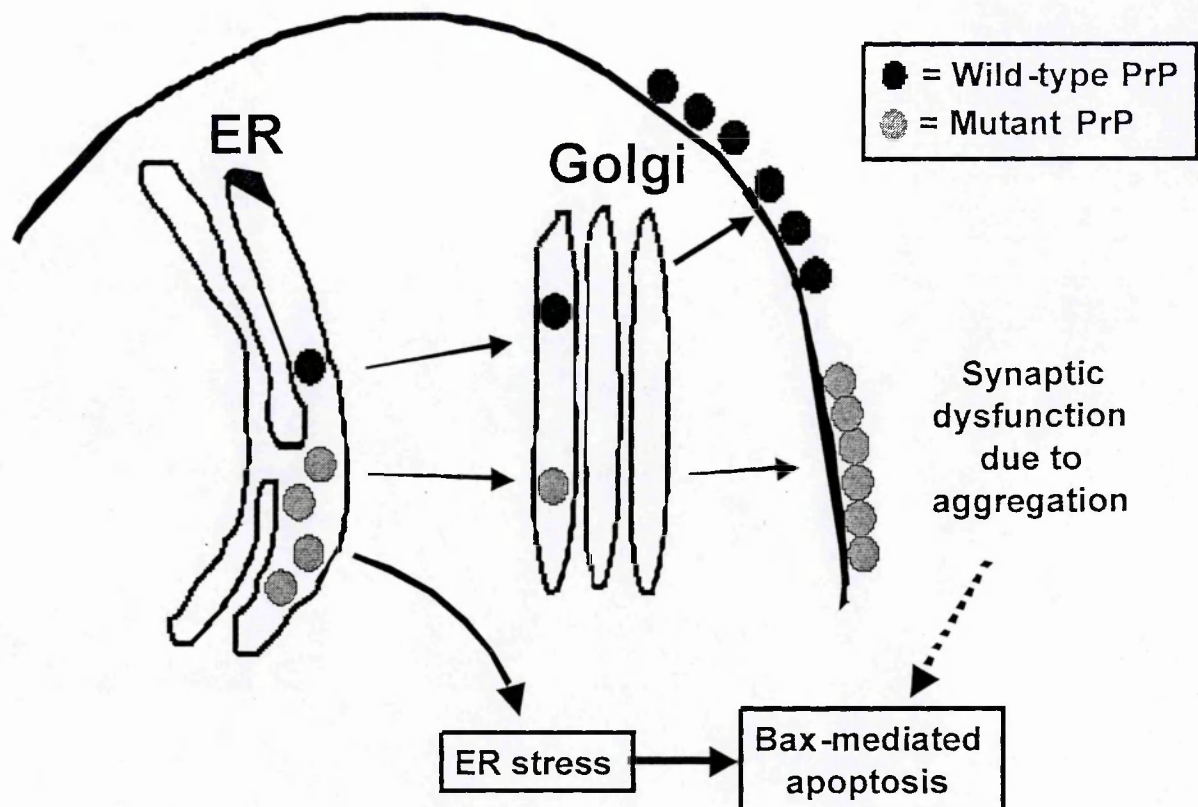


Figure 4.1: Possible pathway for cellular damage that could be initiated by PrP carrying D177N and PG14 mutations.

Mutant PrP retained in the ER could trigger ER stress response and lead to apoptosis. However, results presented in this thesis strongly argue against the involvement of ER stress. Alternatively it is possible to speculate that neurotoxicity is triggered by molecules that reach the cell surface. Mutant PrPs could interact abnormally with other proteins, thus leading to synaptic dysfunction documented for Tg(PG14). Synaptic dysfunction could consequently induce neuronal death.

A support for the hypothesis that altered forms of PrP may cause synaptic dysfunction comes from studies of differentiated PC12 infected with scrapie prions (Rubenstein et al., 1991). In fact, when compared to controls, these cells have shown a decreased activity of choline acetyltransferase and acetylcholinesterase, accompanied by an increment of choline. On the basis of these results the model developed in this thesis will be used to investigate the effect of mutant PrP expression on the activity of some cholinergic pathway-related enzymes.

Finally, to exclude the involvement of ER stress Tg(PG14) mice could be crossed to transgenic mice expressing an ER stress indicator, constructed by fusing XBP1 and venus, a variant of green fluorescent protein (GFP) (Iwawaki et al., 2004). During stress, the spliced indicator mRNA is translated into an XBP1-venus fusion protein, which can be detected by its fluorescence. Tg(PG14) mice could also be crossed to mice transgenic for a GFP carrying an active degradation signal for the proteasome (Lindsten et al., 2003). Administration of proteasome inhibitors to these mice resulted in accumulation of GFP in multiple tissues. Analysing the crossed mice, changes in the amount of GFP are expected if expression of PG14 PrP activate the UPR pathway

Chapter 5: Appendix

5.1 Solutions

5.1.1 Bacterial media

1 L YT: 1 g Tryptone, 5 g Yeast Extract, 5 g NaCl (for plates 15 g of Bacto-agar was added).

5.1.2 Cell culture

Coating:

Poly-L-lysine coating: Plates were incubated for 2 h at 37°C or overnight at room temperature with an appropriate volume of poly-L-lysine solution (Sigma Aldrich Co., St. Louis, Missouri) (0.1 mg/ml diluted in sterile water). After removing poly-L-lysine, plates were washed three times with PBS and air-dried.

Collagen coating: 150 µg/ml of rat-tail collagen (Cohesion Inc., Palo Alto, California) were diluted in 30% ethanol. 25 µl of this solution were added to each well of a microtitre plate and air-dried. Alternatively, 3.5 ml were added to a 100-mm plate.

Media:

PC12 Tet-on: DMEM (Biochrom AG, Berlin, Germany), supplemented with 5% inactivated foetal bovine serum (FBS, Gibco, Life Technologies, Paisley, Scotland), 10% inactivated horse serum (HS, Sigma Aldrich Co., St. Louis, Missouri), 5 mM penicillin/streptomycin (Invitrogen, life

technologies, Carlsband, California), 2 mM L-glutamine (Gibco, Life Technologies, Paisley, Scotland), 200 µg/ml G418 (Gibco, Life Technologies, Paisley, Scotland).

CHO: MEM-α (Gibco, Life Technologies, Paisley, Scotland), supplemented with 10% FBS (Gibco, Life Technologies, Paisley, Scotland), 5 mM penicillin/streptomycin, 2 mM L-glutamine, 200 µg/ml G418. For testing sera, FBS was replaced by HS.

Microglia: DMEM containing 2 mM glutamine, 5 mM penicillin/streptomycin and 10% FBS.

Cells freezing medium: DMEM (or MEM-α), supplemented with 2 mM glutamine, 20% FBS and 10% dimethyl sulfoxide (DMSO, Sigma Aldrich Co., St. Louis, Missouri).

Neuronal differentiation: DMEM supplemented with 1% HS, 5 mM penicillin/streptomycin, 2 mM L-glutamine, 200 µg/ml G418, 80 µg/ml hygromycine.

Serum inactivation: FBS or HS were incubated for 20 min at 56°C to inactivate complement, which can mediate cell lysis.

Splitting cells:

Trypsin/EDTA solution: Trypsin/EDTA stock solution (0.5% Trypsin, 5.3 mM EDTA, Gibco, Life Technologies, Paisley, Scotland) was diluted 10 fold in sterile PBS.

Viability assay:

MTT 10X: 4 mg/mL MTT (Sigma Aldrich Co., St. Louis, Missouri) in Phosphate Buffered Saline (PBS, Gibco, Life Technologies, Paisley, Scotland).

Acidic propanol: 40 mM HCl in propanol.

Crystal violet solution: 0.5% crystal violet (Sigma Aldrich Co., St. Louis, Missouri) in H₂O/methanol solution (4 :1).

Citrate solution: 0.05M Sodium citrate in 47.5% ethanol.

Lysis/loading buffers:

Laemli sample buffer 2X: 100 mM Tris-HCl pH 6.8, 4% (w/v) SDS, Sigma Aldrich Co., St. Louis, Missouri), 20% (v/v) Glycerol, 0.2% (w/v) bromophenol blue, 100 mM dithiothreitol (DTT, Sigma Aldrich Co., St. Louis, Missouri).

Lysis buffer: 10 mM Tris pH 7.4, 100 mM NaCl, 0.5% Igepal Ca-640 (Sigma Aldrich Co., St. Louis, Missouri) and 0.5% Deoxycholic acid (DOC, Sigma Aldrich Co., St. Louis, Missouri).

5.1.3 Western blotting

Laemli Running Buffer 10X: 240 mM Tris, 14% g Glycine (Sigma Aldrich Co., St. Louis, Missouri), 1% SDS.

CAPS Transfer Buffer 10X: 2.4% CAPS (3-(cyclohexylamino)-1-propanesulfonic acid, Sigma Aldrich Co., St. Louis, Missouri), pH 11.

Tris buffer saline (TBS) 10X: 100mM Tris-HCl, pH 7.4, 1.5M NaCl.

TTBS: 0.1% Tween-20 (Sigma Aldrich Co., St. Louis, Missouri) in TBS

5.1.4 Agarose gel electrophoresis

TAE Running buffer 50X: 0.2M Tris Base, 5.7% Acetic acid, 0.05 M EDTA.

5.1.5 RNA analysis

RNase-free water: 500 μ l of Diethyl pyrocarbonate (DEPC, Sigma Aldrich Co., St. Louis, Missouri) in 500 ml water. Autoclaved for 30 min.

Northern blotting:

Denaturing buffer: 0.6% 3-(N-Morpholino) propanesulfonic acid (MOPS, Sigma Aldrich Co., St. Louis, Missouri) containing 32.5% deionized formamide (Sigma Aldrich Co., St. Louis, Missouri), 7% formaldehyde (Sigma Aldrich Co., St. Louis, MO) and 0.0026 μ g/ml Ethidium Bromide (Sigma Aldrich Co., St. Louis, Missouri).

Loading buffer 10X: 0.4% (w/v) bromophenol blue, 50% (v/v) glycerol and 1mM EDTA.

SSC 20X (for capillary elution): 0.3M sodium citrate, 3 M NaCl.

MOPS Running Buffer 10X: 0.2M MOPS, 50 mM sodium acetate, 1 mM EDTA.

Hybridisation buffer: SSC 5X, 5% Dextran Sulfate (Sigma Aldrich Co., St. Louis, Missouri), 0.1% SDS, denatured salmon sperm DNA 100 μ g/ml, 0.05% liquid block (provided by the kit).

Buffer A: 0.1M Tris-HCl, pH 9.5, 0.3M NaCl.

RT-PCR:

Reverse transcriptase buffer 5X (Promega Corporation, Madison, Wisconsin): 250 mM Tris-HCl pH 8.3, 250 mM KCl, 50 mM $MgCl_2$, 2.5 mM spermidine, 50 mM DTT.

5.1.6 Cell imaging

Imunostaining:

PFA: 16% stock solution was purchased by Electron Microscopy Sciences, Washington, Pennsylvania. 4% solutions were made fresh in each experiments.

Blocking buffer: 5% NFDM, 2% FBS in PBS

Propidium iodide staining:

Propidium Iodide mix: 10 µg/ml propidium iodide (Sigma Aldrich Co., St. Louis, Missouri), 500 µg/ml RNase A (Sigma Aldrich Co., St. Louis, Missouri), in PBS.

TUNEL staining:

Saponin solution: 0.1% (w/v) saponin (Sigma Aldrich Co., St. Louis, Missouri), 0.005% (v/v) Tween-20 (Sigma Aldrich Co., St. Louis, Missouri), 1mM EDTA in PBS.

tdT reaction mixture: 5X reaction buffer, 2.5 mM CoCl₂, 20U TdT, 2 nM biotin-16-dUTP.

Blocking buffer: SSC 4X, 0.05 NFDM.

Staining buffer: SSC 4X, 0.001 Triton X-100, 0.05 NFDM.

X-gal staining:

X-gal mixture: 1 mg/ml X-gal (Sigma Aldrich Co., St. Louis, Missouri), 2 mM MgCl₂, 5 mM K₃Fe(CN)₆ and 5 mM K₄Fe(CN)₆ in PBS.

5.2 Antibodies

3F4 monoclonal antibody (epitope 109-112 of human PrP) was a gift from Richard Kascsak and was diluted 1:5,000 and 1:500 for Western blot and immunofluorescence, respectively; P45-66 rabbit polyclonal antibody (raised against residues 45-66 of moPrP) was a gift from David A. Harris (Washington University, St. Louis, Missouri) and was used 1:1,000 or 1:250 for Western blot and immunofluorescence, respectively. Mouse monoclonal antibody 98A3, kindly provided by Jan P.M. Langeveld (CIDC-Lelystad), was diluted 1:5000 for Western blot. Anti-actin monoclonal antibody (MAB1501R, Chemicon International, Temecula, California) was diluted 1:10,000 for Western blot. Anti-CHOP antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, California) was used 1:200 or 1:50 for Western blot and immunofluorescence, respectively. Anti-giantin monoclonal antibody and anti-calnexin rabbit polyclonal antibody were a gift from Professor Roberto Sitia (San Raffaele Scientific Institute, Dibr, Milan) and were used 1:1,000 and 1:100, respectively for immunofluorescence. Anti-mouse and anti-rabbit IgG peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, California) were diluted 1:5,000. Alexa 488(green)-goat anti-mouse IgG and Alexa546(red)-goat anti-rabbit IgG (Molecular probes Inc., Eugene, Oregon) were diluted 1:500.

5.3 Transgenic mice

Transgenic mice expressing wild-type and PG14 mouse PrPs tagged with an epitope for the monoclonal antibody 3F4 were previously generated (Chiesa et al., 1998). In this study transgenic mice of the Tg(WT-E1) line that express 4 times the endogenous PrP level, referred throughout the text as Tg(WT), as well as Tg(PG14-A3) mice expressing PG14 PrP at the endogenous level, and Tg(PG14-C) mice expressing 0.3 times the endogenous level, were used. These transgenic mice were originally generated on a C57BL/6J X CBA/J hybrid and were subsequently bred with the Zürich I line of PrP^{0/0} mice (C57BL/6J X 129 background) (Bueler et al., 1992), resulting in animals that express Tg PrP but not endogenous mouse PrP. PrP^{0/0} mice were non-transgenic littermates of Tg(WT) and Tg(PG14) animals.

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